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THE REGULATION OF PHYTOCHROME INTERACTING FACTOR1 AND ITS ROLE IN LIGHT SIGNALING

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THE REGULATION OF PHYTOCHROME INTERACTING FACTOR1 AND ITS ROLE IN LIGHT SIGNALING

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In memory of my father and my uncle,

Luis F. Castillón

and

Raul F. Castillón

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THE REGULATION OF PHYTOCHROME INTERACTING FACTOR 1 AND ITS ROLE IN LIGHT SIGNALING

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Plants modulate their growth and development according to the prevailing light conditions. To detect light signals plants have an array of photoreceptors including the phytochromes which monitor the red and far-red light regions of the light spectrum. Phytochromes regulate gene expression in response to light in part by physically interacting with nuclear-localized bHLH transcription factors called PHYTOCHROME INTERACTING FACTORS (PIFs). PIFs are known to function as negative regulators of photomorphogenesis. Here we show that PIF1, the PIF family member with the highest affinity for phys, is degraded after pulses or continuous red, far-red or blue light in a phytochrome dependent manner. In etiolated seedlings, phyA plays a dominant role in regulating the degradation of PIF1 after a pulse of red, far-red or blue light; while phyB, phyD and other phys also influence PIF1 degradation after prolonged illumination. PIF1 interacted with phyA and phyB in a blue light-dependent manner, and the interactions with phys are necessary for the light-induced degradation of PIF1. In response to red, far-red or blue light treatments PIF1 is rapidly phosphorylated, poly-ubiquitinated and degraded via the ubiquitin/26S proteasomal pathway. In addition, we show that PIF1 negatively regulates photomorphogenesis at the seedling stage. The overexpression of a light-stable truncated form of PIF1 causes constitutively photomorphogenic phenotypes in the dark. *pif1* seedlings displayed more open cotyledons and slightly reduced hypocotyl length compared to wild type under diurnal (12h light/12h dark) blue light conditions. Double mutant analyses demonstrated that *pif1phyA*, *pif1phyB*, *pif1cry1* and *pif1cry2* have enhanced cotyledon opening compared to the single photoreceptor mutants under diurnal blue light conditions. Taken together, these data suggest that PIF1 functions as a negative regulator of photomorphogenesis and that light-activated phys induce the

degradation of PIF1 through the ubi/26S proteasomal pathway to promote photomorphogenesis.

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INTRODUCTION[1]

Light is a key environmental factor that regulates plant growth and development. Light is involved in controlling multiple responses throughout the plant's life cycle. In some species of angiosperms, including *Arabidopsis*, light is needed to induce seed germination. After germination, the seedling growing in subterranean darkness is said to be "etiolated". Etiolated growth is characterized by a rapid elongation of the hypocotyl (seedling stem) which allows the seedling to reach photosynthetic light as soon as possible. The cotyledons (seedling leafs) of an etiolated seedling are closed to avoid friction with the soil particles and their pale yellow color indicates the absence of chlorophyll. The developmental program corresponding to growth in darkness is called skotomorphogenesis, in contrast to photomorphogenesis in which plant development is regulated by light.

The most striking morphological change triggered by light occurs when an etiolated seedling reaches the surface and is exposed to light for the first time. This process is called de-etiolation, and is characterized by a rapid change in gene expression that inhibits hypocotyl elongation, triggers cotyledon opening, chlorophyll production, and maturation of the chloroplast and photosynthetic apparatus. These adaptations will ultimately enable the seedling to perform photosynthesis. In the juvenile and adult stages of the plant life cycle, light continues to govern plant development, for example: growth towards the source of light (phototropism), rapid elongation if kept in shade (shade avoidance response), perception of the seasons (photoperiodic responses), perception of

[1] Significant portions of this chapter appear in the article:

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day/night cycles (circadian rhythms), chloroplast movement directed by light, stomatal opening and flowering time (Kendrick and Kronenberg, 1996).

Plants have evolved molecular mechanisms to detect the presence or absence of light as well as the duration, wavelength and intensity of incident light. Plants detect light through an array of photoreceptors, each responding to specific regions of the light spectrum. The phytochrome family responds to the red and far-red regions, while phototrophins and cryptochromes respond to the UV-A and blue light regions (Quail, 2002). In addition, there is evidence for the presence of unidentified UV-B light receptor(s), UV-A and blue light receptor(s) and green light receptor(s) (Imaizumi *et al.*, 2005; Somers *et al.*, 2004; Crosson *et al.*, 2003; Folta, 2004). It is now widely believed that the plant's physiological responses to light are achieved by the integration of signals coming from the different photoreceptors. The idea that there is crosstalk between the different photoreceptor systems is supported by the direct interaction of members of the phytochrome and cryptochrome families (Mas *et al.*, 2000; Ahmad *et al*., 1998) and the identification of shared downstream signaling components for cryptochrome and phytochrome pathways (Duek and Frankhauser, 2003).

PHYTOCHROME MEDIATED LIGHT SIGNALING

Phytochromes are the photoreceptors that monitor the red and far-red light regions of the spectrum. Phytochromes control many biological responses including seed germination, initiation of photomorphogenesis (de-etiolation), shade avoidance, and flowering (Quail, 1994). In angiosperms, phytochromes are classified in to four subfamilies: phyA, phyB/D, phyC/F and phyE. The phytochrome family in *Arabidopsis thaliana* is composed of five members (phyA, B, C, D, E). Monocots lost the phyE subfamily and contain only one phyB paralog presenting only phyA, B, C (Matthews and

Sharrock, 1997). In rice and sorghum phytochromes are present as single-copy genes; while in maize each phytochrome gene is duplicated (Gaut, 2001).

In *Arabidopsis* phenotypical analysis of single phytochrome mutants revealed that the different family members have unique as well as overlapping photosensory characteristics and biological functions. The phytochrome family members act in relationship to each other by: 1) redundantly monitoring the same light signals and eliciting the same physiological response; 2) monitoring the same light conditions but regulating diverse physiological response; 3) monitoring different light signals and regulating the same responses (Quail, 2002).

All phytochrome family members are activated by red light signals. phyA is the only phytochrome family member activated by far-red-light-rich signals or by continuous monochromatic far-red light (Smith, 2000; Whitelam *et al*., 1998). phyA is the most abundant phytochrome in dark-grown seedlings. It is involved in the initiation of photomorphogenesis and is the only family member that is rapidly degraded in response to light. Microarray expression profiles have revealed that a number of transcription factors are early targets induced by phyA activation by far-red light (Tepperman *et al*., 2001). In light-grown plants, phyB is the most abundant phytochrome, it is also involved in seedling de-etiolation but it is activated by red–light-rich signals or continuous monochromatic red light (Quail, 2002). phyC is a weak red light sensor, that modulates the function of other phytochrome family members, there is evidence indicating that it might be involved in blue light sensing (Franklin *et al*., 2003). phyC requires the presence of phyB to function (Monte *et al*, 2003). phyD and phyE have been implicated to act together with phyB in controlling several responses including the shade avoidance response (Devlin *et al*., 1999).

Phytochromes are active as homodimers and heterodimers *in vivo* (Sharrock and Clack, 2004) and each subunit consists of a ~125 kDa polypeptide covalently linked to an

open-chain tetrapyrrol chromophore, phytochromobilin (Fairchild *et al*., 2000). Phytochrome exists in two reversible conformations that have different spectroscopic characteristics: the red light absorbing Pr form (biologically inactive), and the far-red light absorbing Pfr form (biologically active) (Quail, 1994; Schafer and Bowel, 2002). For many years, phytochrome was believed to be a cytosolic protein, but recently it was determined that Pfr form of phytochrome translocates into the nucleus after light activation (Kircher *et al*., 2002; Sakamoto and Nagatani, 1996). Red light induces a Pr to Pfr conformational shift that exposes a nuclear localization signal, and promotes translocation of the Pfr form into the nucleus (Chen *et al*., 2005). It has been demonstrated that nuclear translocation is necessary for the majority of the biological functions of phyA and phyB (Huq *et al*., 2003; Matsushita *et al*., 2003; and Hiltbrunner *et al*., 2006). However, phyA also shows distinct cytosolic functions, which are independent of nuclear translocation, such as controlling negative gravitropism in blue light and enhanced phototropism in red light (Rosler *et al*., 2007). In the nucleus phytochromes localize in speckles or nuclear bodies (Kircher *et al*., 2002), and trigger a transcription cascade that leads to the regulation of light responsive genes. Approximately 2500 genes in *Arabidopsis* (10% of the genome) are regulated by phytochrome (Quail, 2002). Evidence indicates that one of the ways by which phytochromes are able to coordinate this massive response to light by directly interacting with transcription factors of the basic-helix-loop-helix (bHLH) superfamily.

CRYPTOCHROME MEDIATED LIGHT SIGNALING

Cryptochromes (crys) are blue light receptors that are found both in animals and plants. Most plant cryptochromes are 70 to 80 kD flavoproteins, with distinct amino and carboxy terminal domains. The amino terminal domain contains conserved binding sites for two chromophores, a flavin (FAD) and a pterin (methenyltetrahydrofolate). The function of the carboxy terminal domain is unclear, but its overexpression produces a

consitituve photomorphogenic response in darkness similar to COP1 phenotype. Possibly by mimicking the conformation of light activated cryptochrome (Yang *et al*., 2000).

Most plant species contain multiple cryptochromes. *Arabidopsis* has three cryptochromes denominated cry1 (Hoffman *et al*., 1996), cry2 (Lin *et al*., 1996) and cry3 (Kleiner *et al*., 2003). Cryptochromes regulate gene expression in response to blue light and participate in the entrainment of the circadian clock. cry1 and cry2 together control the expression of 10 to 20% of *Arabidopsis* genome in response to blue light. The role of cryptochromes in de-etiolation includes inhibition of stem elongation and cotyledon expansion by blue light (Cashmore *et al*., 1999). Cryptochromes also regulate the initiation of flowering in response to day length (Lin 2000), anthocyanin accumulation and stomatal opening. The responses triggered by cryptochromes and phytochromes overlap, since both photoreceptor families participate in de-etiolation and flowering.

CRY1 and *CRY2* genes were identified in mutant screens by their characteristic elongated phenotype in response to blue light treatment (Ahmad and Cashmore, 1993). *Arabidopsis* seedlings overexpressing cry1 or cry2 also present shorter hypocotyls when grown in blue light (Lin *et al*., 1993). cry1 is mainly responsible for de-etiolation in high blue light conditions, while cry2 accounts for de-etiolation under low blue light intensities. There seems to be a functional redundancy in cry1 and cry2 function since *cry1cry2* double mutants have a more severe de-etiolation phenotype than the single mutants. Another characteristic of photomorphogenesis is the accumulation of anthocyanins in response to light. In the *cry1* mutant this accumulation does not occur indicating that anthocyanin accumulation is mainly a blue light response (Ahmad *et al*., 1995). cry2 plays an important role in the photoperiod dependent induction of flowering and *cry2* mutant seedlings exhibit late flowering (Guo *et al*., 1998). The *cry2* mutant has impaired cotyledon opening when exposed to low-irradiance blue light.

Regardless of the presence or absence of light cry2 is constitutively imported to the nucleus (Guo *et al*., 1999; Kleiner *et al*., 1999) where it interacts with chromatin (Cutler *et al*., 2000). It is unclear if the binding is direct or if cry2 associates with chromatin bound proteins, to regulate transcription. In plants expressing GFP tagged cry2, exposure to blue light triggers the formation of distinct punctuate structures or speckles. cry2 is light labile and is degraded in response to blue light. The localization of cry1 seems to differ from cry2, since it accumulates in the nucleus primarily in the dark and seems to be in the cytosol in the light. This can indicate that cry1 might be imported to the nucleus in the dark but exported or remain in the cytosol in response to light treatment.

Cryptochromes are phosphorylated in response to light. It has been reported that oat phyA protein phosphorylates cry1 *in vitro* (Ahmad *et al*., 1998), another report claims that cry1 is autophosphorylated in response to blue light when expressed in insect cells (Shalitin *et al*., 2003). cry2 phosporylation is detected in etiolated seedlings after being exposed to blue light. The amount of phosphorylated form of cry2 to increase, and this form is degraded.

The direct interaction of cryptochromes with COP1 (Yang *et al*., 2000; Yang *et al*., 2001) has led scientists to believe that cryptochromes might function by controlling the stability of other proteins. It has led scientists to believe that crys posses a very short signal transduction cascade, and that this fact probably explains why very few components of the cascade have been identified so far. Some of these components are shared with phytochrome signaling pathway, strengthening the concept that there are interconnections between both pathways.

CRYPTOCHROME AND PHYTOCHROME PATHWAYS

Studies have shown that cryptochromes and phytochromes together regulate photomorphogenic development, floral initiation and the entrainment of the circadian clock (Casal and Mazzella, 1998; Somers *et al*, 1998; Neff and Chory, 1998). The phytochromes monitor mainly the red and far-red light regions of the spectra but they absorb a wide range of visible light wavelengths including blue light. There is an interaction between the red/far-red and blue light signaling pathways. The direct interaction of phyB and cry2 has been reported (Mas *et al*., 2000). Upon blue light exposure, cry2 forms punctuate structures or speckles that colocalize with phyB. cry1, cry2 and phyB are regulators of flowering time, while the phyB mutant flowers early, the cry2 and the phyA mutants are late flowering. Studies involving the *cry2phyB* double mutant have revealed that phyB and cry2 play antagonistic roles in flowering initiation. phyB mediates a red-light inhibition of floral initiation while cry2 inhibits phyB function in a blue-light-dependent manner. There are reports that cry1 can interact with phyA via its C terminal domain in a yeast two-hybrid screen (Ahmad *et al*., 1998) and indirectly with phyB through COP1. The cryptocrome and phytochrome pathways also share in common certain factors, like HFR1.

PHYTOCHROME INTERACTING FACTORS

The bHLH superfamily of transcription factors in *Arabidopsis* consists of approximately 150 members divided into 21 subfamilies (Heim *et al*., 2003; Toledo-Ortiz *et al*., 2003). Members of the bHLH superfamily are characterized by the bHLH signature domain. The bHLH domain consists of two distinct regions: an approximately 15 amino acid basic region involved in binding to the target DNA, and the helix-loop-helix region, consisting of approximately 60 amino acids involved in dimerization. bHLH transcription factors bind DNA as dimers, either as homodimers or heterodimers.

The basic region of each member of the dimer binds DNA at a cis-acting regulatory element found in the promoter region of target genes. The majority of the bHLH transcription factors bind at cis-elements called E-boxes (5'-CANNTG-3'). There are different types of E-boxes depending on the central two nucleotides, for example G-boxes are (5'-CACGTG-3'). As is the case with animal bHLH transcription factors (Littlewood and Evans, 1988), it is probable that sequences surrounding the E-box also help determine binding specificity. In the case of bHLH factors that form heterodimers with multiple partners, each different pair would bind to slightly diverse regulatory sequences conferring specificity of action.

Six members of bHLH subfamily 15 have been identified as capable of binding to phytochromes. Not all members of subfamily 15 bind to phytochromes, even if they have high sequence similarity. The known phytochrome binding factors are denominated phytochrome interacting factors (PIFs) and are PIF1, PIF3, PIF4, PIF5, PIF6, and PIF7. PIF3 was the first PIF to be identified through a yeast two-hybrid screen using the C-terminal domain of phyB as bait. Subsequently, PIF4 was isolated from genetic and reverse genetic approaches (Huq and Quail, 2002). PIF1, PIF5, PIF6 and PIF7 were identified by sequence homology to PIF3 (Huq *et al*., 2004; Khanna *et al*., 2004). These were also given the alternative name of PIF3-like factors (PILs) in such a way that PIF1, PIF5 and PIF6 are also called PIL5, PIL6 and PIL2, respectively (Yamashino *et al*., 2003).

Through their basic domains PIF1, PIF3 and PIF4 bind to a subtype of the E-box called the G-box (5'-CACGTG-3') (Huq and Quail, 2002; Huq *et al*, 2004; Martinez-Garcia *et al*., 2000). The HLH region allows PIFs to form homodimers and/or heterodimers. PIF3 can homodimerize, but can also heterodimerize with PIF4. Both the PIF3-PIF3 homodimer and the PIF3-PIF4 heterodimer can bind to the G-box DNA sequence elements (Toledo-Ortiz *et al*., 2003). PIFs can also heterodimerize with other

non-PIF bHLHs. PIF3 heterodimerizes with HFR1 (long hypocotyl in far-red), an atypical bHLH factor, functioning positively in far-red and blue light signaling pathways (Duek and Frankhauser, 2003; Fairchild *et al*., 2000). However, the functional significance of these heterodimerizations is not clear.

PIFs can interact with different phytochrome family members with differential affinities. PIF3 selectively interacts with the Pfr forms of both phyA and phyB (Zhu *et al*., 2000; Shimizu-Sato *et al*., 2002; Ni *et al*., 1999). PIF1, PIF3 and PIF6 have a stronger affinity for the Pfr form of phyB compared to PIF4 and PIF5. Sequence alignments showed that PIFs share in common a conserved sequence motif, designated as Active Phytochrome Binding (APB) motif (Khanna *et al*., 2004). This motif is located in the N-terminal region of the transcription factor and is necessary and sufficient for binding to the biologically active, Pfr form phyB. Site-directed mutagenesis revealed that four amino acid residues (ELxxxxGQ) common in all the PIFs are critical components of the APB motif**.** However, despite the presence of these four residues several other closely related bHLH proteins in subfamily 15 (e.g. PIL1) did not show interaction with phyB in *in vitro* co-IP assays (Khanna *et al*., 2004). This might be due to the fact that sequences within and around the APB motif might also be critical in determining affinity for phyB.

Only two members of the PIF family, PIF1 and PIF3, have been found to bind to the Pfr form of phyA (Shimizu-Sato *et al*., 2002; Ni *et al*., 1999; Huq *et al*., 2004). PIF1 showed much stronger affinity for phyA than PIF3. However, the sequence motif present in these PIFs that is necessary for phyA interaction is not conserved as the APB motif. In PIF3, a putative Active phyA (APA) binding motif is necessary for its interaction with phyA (Al-Sady *et al*., 2006). This domain has been shown to be necessary for the red light-induced degradation of PIF3 *in vivo*. Site-directed mutagenesis experiments within this domain have shown that two phenylalanine residues (F203 and F209 in PIF3) are critical in the interaction between PIF3 and phyA. Although a similar motif is present in

PIF1, site-directed mutagenesis of the two corresponding phenylalanine residues in PIF1 did not reduce interaction between PIF1 and phyA. Furthermore, in PIF1 several deletions of critical amino acids (residues 85 to 94, 84 to 117 and 118 to 160) dramatically reduced its interaction with the Pfr form of phy A (Shen *et al*., 2008). Site-directed mutagenesis revealed that Leucine 95 and Asparagine 144 are required for PIF1 to bind to the Pfr form of phyA (Shen *et al*., 2008). Other proteins that have been shown to interact with phyA, such as PKS1 and NDPK2, (Frankhauser *et al*., 1999; Choi *et al*., 1999) do not share any sequence homology to PIFs. Considering this evidence, the APA motif might not be a conserved domain and further isolation and characterization of additional factors that selectively bind to the Pfr form of phyA is necessary to determine if a conserved APA motif really exists.

BIOLOGICAL FUNCTIONS OF PIFS

Although PIFs are highly similar in sequence and overall motif structure, the monogenic *pif* mutants show unique as well as common morphological phenotypes in light signaling pathways. This suggests that they do not act redundantly, as is the case for many gene families, and that they have overlapping as well as distinct biological functions (Huq and Quail, 2002; Huq *et al*., 2004; Oh *et al*., 2004, Monte *et al*., 2004; Kim *et al*., 2003).

PIF1 acts as a repressor of light-induced seed germination, light-induced inhibition of hypocotyl elongation, hypocotyl negative gravitropism in the dark and chlorophyll accumulation in light. *pif1* mutants germinate after far-red light exposure, indicating a disruption in the maintenance of dormancy (Oh *et al*., 2004). *pif1* mutants also exhibit slightly shorter hypocotyl length under alternating far-red light and dark cycles and reduced hypocotyl negative gravitropism in the dark compared to wild type (Oh *et al*.,

2004). *pif1* seedlings exhibit photooxidative damage (bleaching) and fail to green when dark-grown seedlings are transferred to light (Huq *et al*., 2004). This phenotype is more severe if the seedlings are kept in darkness for longer times before being transferred to light. The *pif1* bleaching phenotype is the result of the accumulation of 4-6-fold more protochlorophyllide, a phototoxic chlorophyll precursor, over wild type levels. Furthermore, *pif1* mutants accumulate higher levels of chlorophyll when young dark-grown seedlings are transferred to light, suggesting that PIF1 acts as a negative regulator of chlorophyll biosynthesis (Huq *et al*., 2004).

The biological functions of PIF3 are to control morphological phenotypes and biochemical pathways in response to light. The initial characterization of PIF3 involved antisense lines that showed a hyposensitive phenotype under continuous red light, suggesting that PIF3 functions positively in controlling photomorphogenesis (Ni *et al*., 1998). However, several independently isolated alleles of *pif3* mutant seedlings (obtained by T-DNA insertion and fast-neutron-induced deletion) showed shorter hypocotyls and more expanded cotyledons than wild type seedlings under continuous red light, suggesting that PIF3 functions as a negative regulator of morphological phenotypes under red light (Monte *et al*., 2004; Kim *et al*., 2003). By contrast, PIF3 functions positively in chloroplast development and greening processes during the initial hours of de-etiolation, as *pif3* seedlings show lower than wild type levels of chlorophyll (Monte *et al*., 2004; Kim *et al*., 2003). PIF3 also acts positively in the light-induced accumulation of anthocyanin (Kim *et al*., 2003). However, because PIF3 is so closely related to PIF1, it is possible that *pif3* seedlings also undergo photooxidative damage (a phenotype displayed by *pif1* mutants) under light conditions, reducing the ability of these seedlings to synthesize chlorophylls. Further experiments are necessary to distinguish whether the reduced chlorophyll content of the *pif3* mutant is due to PIF3's positive role in these pathways or due to the photoxicity effects under these light conditions.

PIF4 promotes hypocotyl elongation by functioning as a negative regulator in the phyB-mediated inhibition of hypocotyl elongation. Under continuous red light, *pif4/srl2* seedlings show shorter hypocotyls and expanded cotyledons compared to wild type seedlings (Huq and Quail, 2002). *pif5* seedlings also show hypersensitive phenotype similar to that of *pif4* seedlings when exposed to continuous red light, suggesting that PIF5 also functions as a negative regulator of phyB signaling (Fujimori *et al*., 2004). The biological function of PIF6 is unknown. Analysis of *pif3*, *pif4* and *pif7* double mutants, has shown that the hypersensitivity to red light is additive, indicating that these factors act redundantly as negative regulators of photomorphogenesis (Leivar *et al*., 2008). *pif4* and *pif5* seedlings do not display any phenotype under far-red light (phyA response) or in the dark. Therefore, it appears that the major biological function of these factors is to negatively regulate light-induced photomorphogenic development.

All PIFs have been shown to interact with the central clock component APRR1/TOC1 using Y2H assays (Yamashino *et al*., 2003). Moreover, both PIF4 and PIF5 mRNA and protein levels are strongly regulated by the circadian clock, and this regulation is involved in controlling the rhythmic growth pattern of *Arabidopsis thaliana* seedlings under day-night cycles (Yamashino *et al*., 2003; Fujimori *et al*., 2004; Nozue *et al*., 2007). These data suggest that PIF4 and PIF5 function in the circadian clock. The circadian clock defects for other *pif* mutants have not been shown yet.

IDENTIFICATION AND LIGHT-REGULATION OF DIRECT TARGET GENES OF PIFS

Because PIFs are transcription factors capable of directly binding to both potential target gene promoters and photoactivated phytochromes, they are ideal to investigate the mechanisms of light regulated gene expression. The interaction of photoactivated phyB

with DNA-bound PIF3 suggested a model involving the direct regulation of gene expression by phytochromes in response to light (Martinez-Garcia, 2002; Quail 2002). However, conclusive evidence in favor of this or any other model is still absent due in part to the lack of known direct target genes of PIFs. With the recent optimization of the chromatin immunoprecipitation (ChIP) assay, direct target genes of PIF1 and PIF3 have been identified. Using ChIP and *in vitro* gel-shift assays, it was shown that PIF1 directly activates two genes in the dark, *GAI* and *RGA*, that encode DELLA proteins involved in GA mediated regulation of seed germination (Oh *et al*., 2007). ChIP assays also showed that PIF3 binds six gene promoters *in vivo* that are involved in controlling anthocyanin biosynthesis (Shin *et al*., 2007). Both PIF1 and PIF3 control other pathways involved in photomorphogenesis in addition to seed germination and anthocyanin biosynthesis. Moreover, there are no known targets of PIF4, PIF5 and PIF6. Therefore, the identification and characterization of genomic targets of PIFs using the ChIP-chip technique is necessary to fully understand how these factors control photomorphogenesis (Hudson and Snyder, 2006).

The identification of PIF target genes allowed further understanding of the role of PIFs in the light-regulation of these genes. The expression of *GAI* and *RGA*, two direct target genes of PIF1, is down regulated by light, consistent with the light-induced degradation of PIF1 (Oh *et al*., 2007, Shen *et al*., 2005, Oh *et al*., 2006). Similarly, the direct target genes of PIF3 (e.g., *CHS, CHI, F3H, DFR* and *LDOX*) are down regulated by far-red light (Shin *et al*., 2007). However, the PIF3 protein level is not reduced by light under these conditions (Shin *et al*., 2007). PIF3 binding to these promoters is also not regulated by light. In addition, the differences in expression of the above genes were shown only under continuous far-red light without any dark control, preventing evaluation of the relative effect of light on the expression of these genes. These results are not consistent with previous reports that PIF3 is degraded under both red and far-red light conditions (Monte *et al*., 2004, Shin *et al*., 2007, Park *et al*., 2004). Therefore, it is

still not clear how PIF3 directly controls these genes to promote anthocyanin biosynthesis in response to light.

PIF3 has also been implicated in rapid gene expression in response to light. Microarray analysis showed that expression of several genes is compromised in the *pif3* mutant compared to wild type within one hour of red light exposure (Monte *et al*., 2004). Of the genes that showed strong PIF3-dependent regulation, several are photosynthesis or chloroplast related, zinc finger transcription factors, and RNA polymerase sigma factor E, which might regulate the chloroplast genome. However, the expression of these genes might be indirectly regulated by PIF3, as direct binding of PIF3 to these promoters has not been shown. It appears that PIFs can activate gene expression in the dark and that the light-induced degradation of PIFs might reduce expression of certain target genes. However, PIFs might also be involved in the regulation of gene expression in response to light. Further investigations are necessary to determine whether PIFs are involved in light-regulated gene expression.

Another way in which PIFs have been proposed to function as negative regulators of photomorphogenesis is by directly controlling the levels of the photoreceptors (Leivar, 2008). Under prolonged red light PIF3, PIF4 and PIF7 were found to maintain low phyB protein levels and thus modulate photomorphogenesis.

REGULATION OF PIFS' FUNCTION

Although PIFs are transcription factors capable of activating and/or repressing gene expression, only PIF1 has been shown to have transcriptional activation activity *in vivo* (Huq *et al*., 2004). Strikingly, this activity is reduced in light in a phytochrome-dependent manner. Furthermore, PIF1, PIF3, PIF4 and PIF5 proteins are rapidly degraded in

response to light signals (Monte *et al*., 2004; Nozue *et al*., 2007; Shen *et al*., 2005, Oh *et al*., 2006; Park *et al*., 2004; Bauer et a., 2004). Treatment with proteasomal inhibitors prevented degradation, providing evidence that PIFs are degraded by the ubiquitin-26S proteasomal pathway. The half-life for PIF1 and PIF3 is ~10-15 min under red light, suggesting that these factors might function transiently during the dark to light transition (Shen *et al*., 2005; Bauer *et al*., 2004). It has been determined that phyA, phyB and phyD are necessary for the light-induced degradation of PIF3, while COP1 (Constitutively photomorphogenic 1), another negative regulator of light signaling, is necessary for the stability of PIF3 in the dark (Bauer *et al*., 2004). COP1 is a nuclear E3 ligase that represses photomorphogenesis in the darkness by selecting for degradation positive acting factors, such as HY5. Because the major biological function of PIFs is negative regulation of photomorphogenesis, it is not surprising that light negatively regulates PIFs function through phytochromes to promote photomorphogenesis (Huq, 2006).

While rapid degradation of PIF3 led to the conclusion that it functions transiently in light signaling pathways (Bauer *et al*., 2004), subsequent studies have shown that PIF1, PIF3, PIF4 and PIF5 re-accumulate in the dark during recurring light-dark cycles (Monte *et al*., 2004, Nozue *et al*., 2007, Shen *et al*., 2005). The recurring expression of PIF4 and PIF5 has been shown to control rhythmic growth pattern of *Arabidopsis thaliana* seedlings under day-night cycles (Nozue *et al*., 2007). Therefore, PIFs might fine tune photomorphogenic development throughout the plant life cycle.

Although the mechanism of light-induced PIF degradation is still unknown, a recent pivotal article shed some light on the initial steps, showing that PIF3 is phosphorylated in response to light signals in a phytochrome-dependent manner, and the phosphorylated form is rapidly degraded in light (Al-Sady *et al.*, 2006). Strikingly, direct physical interaction with phyA and phyB is necessary for the light-induced phosphorylation and subsequent degradation of PIF3. Mis-sense mutations in both the APA and APB domains

of PIF3 eliminated direct physical interactions with phyA and phyB, respectively. This mis-sense mutant, which does not interact with phytochromes, is not phosphorylated and is therefore stable under light conditions. These data suggest that the first step in the light-induced degradation of PIF3 is the phosphorylation of PIF3 after direct physical interaction with phytochromes. Work in other systems has shown that many substrates of the ubiquitin-26S proteasome pathway are phosphorylated before degradation. Therefore, PIFs might be phosphorylated in response to light signals in a phytochrome-dependent manner, and the phosphorylated form is degraded by the ubiquitin-26S proteasome pathway to remove the negative regulation of photomorphogenesis.

PIFS INVOLVEMENT IN HORMONE SIGNALING

There is mounting evidence suggesting that light signals coordinate with hormone signaling pathways to control photomorphogenesis (Neff *et al*., 2006). The most direct known link between PIFs and hormone signaling is PIF1's involvement in gibberellin (GA) mediated seed germination. PIF1 inhibits seed germination by repressing GA biosynthetic genes and activating GA catabolic genes, resulting in a reduced level of bioactive GA in wild type seeds (Oh *et al*., 2006). In addition to the regulation of GA biosynthesis and catabolism, PIF1 controls GA sensitivity by directly activating the expression of *GAI* and *RGA*, two key DELLA protein genes that function as repressors of GA signaling (Oh *et al*., 2007). Moreover, PIF1 activates ABA biosynthesis to promote seed dormancy. Light signals perceived by phytochromes present in the seed (mainly phyB) induce degradation of PIF1 to promote GA biosynthesis, increase GA sensitivity and decrease ABA biosynthesis to promote seed germination. Involvement of other PIFs in hormone signaling has not been shown. However, the expression of *PIF* genes is regulated not only at the tissue-specific and developmental stage-dependent manner, but also by multiple hormones, biotic and abiotic stress conditions, suggesting that PIFs might function in multiple hormone and stress signaling pathways in different organs.

HFR1 INTERACTS WITH PIF3

HFR1 (long hypocotyl in far red light) is a nuclear basic helix-loop-helix transcription factor which is known to be important in several phyA responses (Fairchild *et al*., 2000) as well as cryptochrome responses (Duek and Frankhauser, 2003). It has strong similarity to PIF3 and PIF4. HFR1 itself can not bind to phytochromes but HFR1 can form heterodimers with PIF3 (Toledo-Ortiz *et al*., 2003) that bind to the Pfr form of phyA and B. It is an atypical bHLH protein since the basic region in the amino terminus presents a deletion relative to PIF3, which probably affects its DNA binding ability (Fairchild *et al*., 2000). *hfr1* mutants are defective in phyA responses having reduced de-etiolation responses in far red light which include hypocotyl elongation, cotyledon unfolding and agravitropism. *hfr1* mutants also present a hyposensitive hypocotyls phenotype under blue light treatments (Duek and Frankauser, 2003). The observation that *phyA* mutants also have a similar reduced de-etiolation response in blue light, lead to the formulation of two alternative hypotheses: it could be possible that the elongated phenotype of *hfr1* mutants was due to the participation of HFR1 in blue light (cryptochrome) signaling, or that it was due to a disruption of phyA signaling cascade. To distinguish between these two possibilities, and test weather HFR1 acted independently in blue light, a double muntant, *phyA hfr1* was obtained. This double mutant showed greater hypocotyl length when grown in blue light, when compared to the single mutants. These observations lead the authors to suggest that HFR1 acts independently of phyA in blue light reception. Another strong indication that HFR1 also participates in the cryptochrome signaling pathway is that the cotyledons of *hfr1* mutants grown in blue light resemble those of *cry1* mutants and not those of *phyA* mutants.

HFR1 is stable under light conditions, and its expression is induced by light. In the darkness, COP1 and SPA1 have been shown to be involved in its phosphorylation and subsequent degradation (Jang *et al*., 2005; Yang *et al*., 2005; Yang *et al*., 2005b). There is evidence that COP1 and SPA1 might cooperatively function as the E3 ligase that

ubiquitinates HFR1. *hfr1* mutant seedlings also present an exaggerated shade avoidance response (Sessa *et al*., 2005), characterized by elongated stems and petioles and small leaves, when grown under low red to far-red ratio light conditions. Transgenic plants expressing a truncated version of HFR1 lacking the first 150 amino acids show exaggerated responses to light even in the darkness (Yang *et al*., 2003).

FUTURE PERSPECTIVES ON PIFS

Although it is well established that PIFs are central players in phytochrome signaling networks, several key questions regarding PIFs still remain unanswered. It remains to be determined whether or not phytochromes can directly phosphorylate PIF3 and possibly other PIFs in response to light. The direct physical interaction of PIFs with photoactive phytochromes is necessary for the light-induced phosphorylation and subsequent degradation of PIF3 (Al-Sady *et al*., 2006). In addition, phyA has been shown to function as a non-conventional serine/threonine kinase (Yeh and Lagarias, 1998). While convergence of these two bodies of research might have a logical outcome, there is increasing evidence suggesting that phytochromes might not directly function as kinases. The putative kinase domain of phytochromes is localized at their C-terminal domain, which is dispensable for at least one phytochrome (e.g., phyB) signaling pathway (Matsushita *et al*., 2006; Krall and Reed, 2000). Therefore, it remains to be determined what kinase phosphorylates PIF3 and possibly other PIFs in response to light. Also it would be important to know what are the factors responsible for recognition and subsequent ubiquitination of the phosphorylated forms of PIFs, such as the E3 ligase. Another question that can be asked is if the other PIFs are phosphorylated and degraded as it is the case with PIF3. Phosphorylation and ubiquitination of transcription factors are common posttranslational modifications (Mayr and Montminy, 2001; Muratani and Tansley, 2003). Often transcription factors are phosphorylated and/or ubiquitinated at the

transcription initiation complex to activate their transcription activation activity as well as to tag them for subsequent degradation by the ubiquitin-26S proteasome pathway (Mayr and Montminy, 2001; Muratani and Tansley, 2003). This allows cells to proportionately respond to the stimuli that activate the transcription factor. Given that PIF3 is necessary for light-induced gene expression, does light control homo-/hetero-dimerization, DNA binding and transcriptional activation activity of PIFs? The combined power of biochemical, molecular genetic and photobiological techniques will help answer these questions.

CHAPTER II

Phytochromes Mediate the Phosphorylation and Degradation of PIF1 in Response to Red and Far-Red Light[2]

SUMMARY

Phytochromes (phys) regulate changes in gene expression in response to red/far-red light signals in part by physically interacting with constitutively nuclear-localized PIFs, phy-interacting bHLH transcription factors. Here we show that PIF1, the member with the highest affinity for phys, is strongly sensitive to the quality and quantity of light. phyA plays a dominant role in regulating the degradation of PIF1 following initial light exposure, while phyB, phyD and possibly other phys also influence PIF1 degradation after prolonged illumination. PIF1 is rapidly phosphorylated and ubiquitinated under red and far-red light before being degraded with a half-life of ~1-2 min under red light. Although PIF1 interacts with phyB through a conserved active phyB binding (APB) motif, it interacts with phyA through a novel APA motif. phy interaction is necessary, but not sufficient for the light-induced phosphorylation and degradation of PIF1. Domain mapping studies reveal that the phy-interaction, light-induced degradation and the transcriptional activation domains are located at the N-terminal 150 amino acid region of PIF1. Unlike PIF3, PIF1 does not interact with the two halves of either phyA or phyB separately. Moreover, overexpression of a light-stable truncated form of PIF1 causes constitutively photomorphogenic phenotypes in the dark. Taken together, these data

[2] Significant portions of this chapter appear in:

Shen, H., Castillón, A., Zhu, L., Majee, M., Downie, B. and Huq, E. (2008). Light-induced phosphorylation and degradation of the negative regulator PHYTOCHROME INTERACTING FACTOR 1 from *Arabidopsis* depends upon its direct physical interactions with photoactivated phytochromes. *Plant Cell* **20**, 1586-602.

suggest that the removal of the negative regulators (e.g., PIFs) by light-induced proteolytic degradation might be sufficient to promote photomorphogenesis.

INTRODUCTION

Environmental light signals regulate growth and development at all phases of a plant's life cycle. Plants have evolved several light receptors: the phytochrome (phy) family of photoreceptors to monitor the red (R)/far-red (FR) region; the cryptochromes (crys), phototropins (phots) and ZTL/FKF1 family of F-box proteins to monitor the UV-A/blue region; and an unidentified receptor to monitor the UV-B region of the spectrum (Lin and Shalitin, 2003; Chen *et al*., 2004; Schaefer and Nagy, 2006). The phy family in Arabidopsis thaliana (PHYA-PHYE) encodes ~125 kDa soluble proteins that can form selective homo- or hetero-dimers between the family members (Mathews and Sharrock, 1997; Sharrock and Clack, 2004). Their photosensitivity relies on the acquisition of a covalently attached bilin chromophore that enables the existence of two inter-convertible forms of phys: the Pr form (biologically inactive) with maximal absorbance in the R region of the spectrum and the Pfr form (biologically active) with maximal absorbance in the FR region of the spectrum. The Pr form is converted to the biologically active Pfr form under R light, and the Pfr form is converted back to the inactive Pr form under FR light (Rockwell *et al*., 2006). The array of photoreceptors allows plants to monitor and respond to a number of parameters of ambient light signals for optimum photomorphogenic development (Schaefer and Nagy, 2006; Whitelam and Halliday, 2007).

Phytochromes in the Pr form can be found predominantly in the cytosol, but they are induced to translocate into the nucleus upon light activation (Kircher *et al*., 2002). Light induces nuclear import of phys via either a conformation change (in phyB) resulting in the unmasking of a nuclear localization signal (NLS) present in its C-terminal domain

(Chen *et al*., 2005), or an association (of phyA) with other proteins containing an NLS (Zhou *et al*., 2005; Hiltbrunner *et al*., 2006; Rösler *et al*., 2007). Light-induced nuclear translocation is necessary for the majority of the biological functions of phyA and phyB (Huq *et al*., 2003; Matsushita *et al*., 2003; Hiltbrunner *et al*., 2006; Rösler *et al*., 2007). However, cytosolic phyA regulates negative gravitropism in blue light as well as red light-enhanced phototropism (Rösler *et al*., 2007). In the nucleus, phys interact with a group of unrelated proteins (Quail, 2007), and initiate signaling cascades that result in changes in expression of ~10% of the genome (Rockwell *et al*., 2006; Jiao *et al*., 2007; Whitelam and Halliday, 2007). However, the primary biochemical mechanism of signal transfer from photoactivated phys to signaling partners is still unknown.

Of all the proteins that are able to interact wih phytochromes, the PHYTOCHROME INTERACTING FACTOR (PIF) family of bHLH transcription factors constitutes the best model for understanding phy-regulated gene expression (Duek and Fankhauser, 2005; Castillon *et al*., 2007; Quail, 2007). Six closely related genes of the *Arabidopsis* bHLH superfamily encode PIF1 and PIF3-PIF7 (Toledo-Ortiz *et al*., 2003; Castillon *et al*., 2007; Quail, 2007; Leivar *et al*., 2008). PIFs interact selectively with the Pfr forms of phys with differential affinities *in vitro*. For example, PIF1 and PIF3 interact with the Pfr forms of both phyA and phyB, while all other PIFs interact with the Pfr form of phyB only (Ni *et al*., 1999; Huq and Quail, 2002; Huq *et al*., 2004; Khanna *et al*., 2004; Leivar *et al*., 2008). Interaction of PIFs with other phys has not been detected. Of all the PIFs, PIF1 has the highest affinity for both phyA and phyB (Huq *et al*., 2004), suggesting that PIF1 plays a critical role in phy signaling.

It has been determined by genetic and photobiological analyses that the PIF family members have distinct, as well as overlapping, biological functions (Castillon *et al*., 2007; Quail, 2007). However, contrary to the initial observation, PIFs act predominantly as negative regulators of phy signaling pathways. PIF3-PIF5 and PIF7 negatively regulate

light-induced suppression of hypocotyl elongation and cotyledon expansion (Huq and Quail, 2002; Kim *et al*., 2003; Fujimori *et al*., 2004; Huq *et al*., 2004; Monte *et al*., 2004; Oh *et al*., 2004). Strikingly, this negative regulation under prolonged R light conditions is correlated with elevated levels of phyB, suggesting that these PIFs regulate phyB protein levels posttranslationally under continuous R light (Monte *et al*., 2004; Al-Sady *et al*., 2008; Khanna *et al*., 2008; Leivar *et al*., 2008). PIF3 also positively regulates chlorophyll biosynthesis and anthocyanin production in light (Kim *et al*., 2003; Monte *et al*., 2004; Shin *et al*., 2007). Recently, PIF3 and PIF4 have been shown to interact with DELLA proteins to coordinately modulate cell elongation (de Lucas *et al*., 2008; Feng *et al*., 2008). PIF1 plays a major role in negatively regulating light-induced seed germination and chlorophyll biosynthesis, as well as playing a minor role in light-induced suppression of hypocotyl elongation and cotyledon expansion. PIF1 regulates gibberellic acid metabolic and signaling genes to suppress seed germination (Oh *et al*., 2006; Oh *et al*., 2007). PIF1 also directly and indirectly regulates chlorophyll biosynthetic genes to optimize the greening process in *Arabidopsis* (Moon *et al*., 2008). These data suggest that although PIFs have the potential to receive the light signals from the photoactivated phys, they have an antagonistic relationship with phys.

The functional significance of the above antagonistic relationship became apparent when it was shown that PIF3 is degraded under both R and FR light conditions in a phy-dependent manner (Bauer *et al*., 2004). Moreover, the transcriptional activation activity of both PIF1 and PIF3 is also reduced under both R and FR light in a phy-dependent manner (Huq *et al*., 2004; Al-Sady *et al*., 2008). Subsequently, it was shown that PIF1 and PIF3-PIF5 are degraded under light through the ubiquitin (ubi)/26S-proteasomal pathway (Monte *et al*., 2004; Park *et al*., 2004; Shen *et al*., 2005; Oh *et al*., 2006; Lorrain *et al*., 2007; Nozue *et al*., 2007; Shen *et al*., 2007). PIF3-PIF5 are also phosphorylated specifically in response to R light, and the phosphorylated form is presumably degraded under light (Al-Sady *et al*., 2006; Lorrain *et al*., 2007; Shen *et al*., 2007). An N-terminal conserved region of PIFs, called the APB (active phyB binding) motif is necessary for

the physical interactions between PIFs and the photoactivated phyB (Khanna *et al*., 2004). Similarly, an APA (active phyA binding) motif within the N-terminal region of PIF3, distinct from the ABP motif, is necessary for the interaction of PIF3 and phyA (Al-Sady *et al*., 2006). Both APA and APB motifs are necessary for the light-induced phosphorylation and subsequent degradation of PIF3. However, despite the fact that PIF1 has the strongest affinity among the PIFs for both phyA and phyB, the functional significance of its direct physical interaction with photoactivated phys has not been demonstrated. Moreover, the early events in FR-induced degradation of PIFs are not yet known. Here we show that although PIF1 has an APB motif similar to other PIFs, it has a different APA motif than PIF3. PIF1 was rapidly phosphorylated and ubiquitinated under both R and FR light, and direct physical interaction of PIF1 with phyA or phyB was necessary for light-induced phosphorylation and degradation. Moreover, overexpression of a light-stable, truncated form of PIF1 generated constitutively photomorphogenic phenotypes in the dark, suggesting that the removal of the negative regulators (e.g., PIFs) by light-induced proteolytic degradation might be sufficient to promote photomorphogenesis.

MATERIALS AND METHODS

Plant Growth Conditions and Phenotypic Analyses

Plants were grown in Metro-Mix 200 soil (Sun Gro Horticulture, Bellevue, WA) under 24 hour light at 24 °C ± 0.5°C. Monochromatic R and FR light sources are as described (Shen *et al*., 2005). Light fluence rates were measured using a spectroradiometer (Model EPP2000, StellarNet Inc., Tampa, FL) as described (Shen *et al*., 2005). Seeds were surface sterilized and plated on Murashige-Skoog (MS) growth medium (GM) containing 0.9% agar without sucrose (GM-Suc) as described (Shen *et al*., 2005). After 3-4 days of moist chilling at 4ºC in the dark, seeds were exposed to 3 hours white light at room temperature in order to satisfy this requirement for the completion of

germination before placing them in the dark for another 4 days. For transgenic plants, the 35S:LUC-PIF1 (LP), 35S:LUC-GFP (LG) lines were generated as described (Shen *et al*., 2005). The 35S:TAP-PIF1 (TP) and 35S:TAP-GFP (TG) transgenic lines were as described (Moon *et al*., 2008). Mutant lines used are as follows: *cop1-6* (McNellis *et al*., 1994), *phyA-211* (Reed *et al*., 1994), *phyB-9* (Reed *et al*., 1993), *phyAB* and *phyABD* in Ler background (Devlin *et al*., 1999), *pif1-2* (Huq *et al*., 2004). For quantitation of hypocotyl lengths, cotyledon areas and cotyledon angles, digital photograph were taken and at least 30 seedlings were measured using the publicly available software ImageJ (http://rsb.info.nih.gov/ij/), and the experiments were repeated at least three times. The photobleaching assays for seedlings, seed germination, hypocotyl negative gravitropism and chlorophyll measurements were performed as described (Huq *et al*., 2004; Shen *et al*., 2005; Shen *et al*., 2007).

Antibody Preparation, Protein Extraction and Western Blotting

The amino acid sequence of PIF1 was examined for unique regions predicted to be of high antigenicity. Peptides (21-mers including a non-coded carboxy-terminal cysteine) were synthesized (United Biochemical Research, Inc., Seattle, WA) corresponding to the region N-terminal to the bHLH section (NH2-EKTNVDDRKRKEREATTTDEC-COOH) and approximately 5 mg of the PIF1 peptide was linked to Keyhole Limpet Hemocyanin (KLH). An additional ~10 mg was linked to agarose gel (Strategic Diagnostics Inc., Newark, DE). KLH-linked peptide was used to immunize 2 New Zealand white rabbits. Serum was prepared following terminal cardiac puncture and affinity purified over the agarose-linked peptide (Strategic Diagnostics Inc., Newark, DE). Affinity purified antibody was stored frozen in aliquots until use.

Four day-old seedlings were either kept in darkness or exposed to R or FR light (amount of light is indicated on individual figures) and incubated in the dark for various times before protein extraction. For detecting TAP-PIF1 and LUC-PIF1 proteins in

transgenic plants, boiling denaturing buffer (100 mM MOPS, pH 7.6, 5% SDS, 10% Glycerol, 4 mM EDTA, 40 mM β-mercaptoethanol) was added at a 1:3 (w/v) ratio before grinding. Protease inhibitor cocktail (1X) (F. Hoffmann-La Roche Ltd, Basel, Switzerland) and 2 mM PMSF were also added during extraction. For detecting native PIF1 in wild type plants, about 0.2 g of tissue were collected and ground in 1 mL of extraction buffer (100 mM Tris-HCl pH 6.8, 20% glycerol, 5% SDS, 80 µM MG132, 20 mM DTT, 1 mM bromophenol blue, 2 mM PMSF, and 1X protease inhibitor cocktail (F. Hoffmann-La Roche Ltd, Basel, Switzerland)) and boiled for 2 min. Total protein supernatants were separated on 8% SDS-PAGE gels, blotted onto PVDF membrane and probed with anti-PIF1 antibody. Another membrane prepared in parallel was challenged with anti-tubulin (T6074, Sigma-Aldrich Co., St. Louis, MO) as a loading control. The Western blot procedure was carried out according to KPL Protein Detector kit (KPL Inc., Gaithersburg, MD) instructions, utilizing a 1:5,000 dilution of anti-PIF1 and a 1:2,500 dilution of the anti-tubulin antibody. Peroxidase-labeled goat anti-rabbit (anti-mouse for tubulin) antibody (KPL Inc., Gaithersburg, MD) in a 1:50,000 dilution was used as secondary antibody. For other immunoblot analyses, the membranes were blocked with 1X TBST plus 0.5% non-fat milk buffer at 4 °C overnight with different primary antibodies as follows: mouse monoclonal anti-PHYA (073D) (1:500), anti-PHYB (B6-B3) (1:500), and anti-ubiquitin (1:700, Santa Cruz Biotechnology, Inc., Santa Cruz, CA) or Rabbit anti-c-MYC (1:800, Sigma-Aldrich Co., St. Louis, MO) and anti-luciferase (1:750) (Promega, Madison, WI). For secondary antibody, peroxidase-labeled goat anti-rabbit antibody (1:4000, Pierce Biotechnology Inc., Rockford, IL) or anti-mouse IgG HRP conjugate (1:3300) (Promega, Madison, WI) was used. Membranes were developed with SuperSignal West Pico Chemiluminescent substrate kit (Pierce Biotechnology Inc., Rockford, IL), and visualized on an X-ray film.

Immunoprecipitation and Alkaline Phosphatase Treatment

For immunoprecipitation (IP) and Calf Intestine Alkaline Phosphatase (CIAP) assays, 4 day-old dark-grown 35S:TAP-PIF1 and 35S:TAP-GFP seedlings were pretreated with MG132 to reduce ubi-mediated protein degradation. Seedling were transferred into MS-suc liquid media containing 30 µM MG132 or equal volume of solvent control DMSO and incubated in the dark for 4.5 hours. Total proteins were extracted from ~0.4 g seedlings (either kept in darkness or treated with 3000 $\mu molm^{-2}$ of Rp or FRp followed by dark incubation) with 1 mL denaturing buffer (100 mM NaH_2PO_4, 10 mM Tris [pH 8.0], 100 mM NaCl, 8 M urea, 0.05% Tween-20, 1X Protease inhibitor cocktail [Roche], 2 mM PMSF, 10 µM MG132, 25 mM β-GP, 10 mM NaF, 2 mM Na-orthovanadate and 100 nM calyculin A) and cleared by centrifugation at 16,000 g for 15 min at 4ºC. TAP-PIF1 was immunoprecipitated from supernatants with Ni-NTA magnetic agarose beads (Qiagen Inc., Valencia, CA) as described (Al-Sady *et al*., 2006). The pellet was resuspended in 100 µL CIAP reaction buffer and then treated either with 100 U CIAP (F. Hoffmann-La Roche Ltd, Basel, Switzerland), with the same amount of boiled CIAP or without enzyme for 60 min at 37ºC. Pellets were washed with PBS buffer, heated at 65ºC in 1X SDS-Laemmli buffer for 5 min and subjected to Western blot analysis with anti-c-MYC or anti-ubiquitin antibody as described above.

Construction of Plasmids and *in vitro*/*in vivo* Co-Immunoprecipitation Assays

The DNA constructs for expressing full-length phyA, phyB, GAD and GAD-PIF1 have been described previously (Huq *et al*., 2004). The phyB deletion constructs are as described (Zhu *et al*., 2000). Various fragments of PIF1 or phyA were amplified by PCR using PfuTurbo enzyme and then cloned into the pET17b vector (EMD Biosciences Inc., Madison, WI) for *in vitro* expression. The specific amino acid mutations in full-length PIF1 were introduced using a site-directed mutagensis kit (Stratagene, La Jolla, CA). Restriction enzyme sites (EcoRI-SalI or EcoRI-XhoI for PIF1 and NdeI-XhoI for phyA) were introduced into the PCR primers, and all the constructs were sequenced completely.

For *in vitro* co-immunoprecipitation assays, all proteins were expressed in the TnT *in vitro* transcription/translation system (Promega, Madison, WI) in the presence of ^{35}S-methionine using the T7 promoter. *In vitro* co-immunoprecipitation experiments and sample preparation were performed as described (Huq *et al*. 2004; Ni *et al*., 1999).

For *in vivo* co-immunoprecipitation assays, seedlings were pretreated with MG132 as described above. Total proteins were extracted from ~0.4 g seedlings (either kept in darkness or treated with 3000 μmolm^{-2} of Rp followed by dark) with 1 mL native extraction buffer (100 mM NaH_2PO_4, pH 7.8, 100 mM NaCl, 0.05% NP-40, 1X Protease inhibitor cocktail [F. Hoffmann-La Roche Ltd, Basel, Switzerland], 2 mM PMSF, 10 μM MG132, 25 mM β-GP, 10 mM NaF, 2 mM Na orthovanadate and 100 nM calyculin A) and cleared by centrifugation at 16,000 g for 15 min at 4ºC. Anti-PIF1 antibody was incubated with Dynabead (Invitrogen Inc., Carlsbad, CA) (20 μL/μg antibody) for 30 min at 4ºC and the beads were washed twice with the extraction buffer to remove the unbound antibody. The bound antibody-beads were added to a total of 500 μg total protein extracts and rotated for another 3 h at 4ºC in the dark. The beads were collected using a magnet, washed three times with wash buffer, dissolved in 1X SDS-Loading buffer and heated at 65ºC for 5 min. The immunoprecipitated proteins were separated on an 8% SDS-PAGE gel, blotted onto PVDF membrane, and probed with anti-phyA, anti-phyB or anti-LUC antibodies as described above.

Cyclohexamide Chase and Luciferase Assays

For cycloheximide chase assays, 4 day-old dark-grown seedlings were pretreated with 50 μM cycloheximide or solvent control DMSO in MS-Suc liquid medium for 3 hours in darkness as described (Shen *et al*., 2005). After pretreatment, the seedlings were exposed to 3000 μmolm^{-2} of R light (Rp) for 1 min, and then kept in darkness before harvesting at different time points indicated in the figures. For luciferase assays, samples were collected in liquid nitrogen and total protein was extracted using 1X Luciferase Cell

Culture Lysis Reagent (CCLR) (Promega, Madison, WI) with 2mM PMSF and 1X complete protease inhibitor cocktail (F. Hoffmann-La Roche Ltd, Basel, Switzerland). Luciferase activity was measured as described (Shen *et al*., 2005).

Construction of Plasmids and Transient Transcription Activation Assays

For transient transcription activation assays, the full-length *PIF1* open reading frame or different fragments were cloned as SmaI – KpnI fragments into pMN6 in-frame with the Gal4 DNA binding domain (DBD) (Huq *et al*., 2004). Full length β-glucuronidase (GUS) cDNA without the stop codon was amplified with Pfu Turbo polymerase (Stratagene, La Jolla, CA) using the SmaI restriction sites at both ends. This fragment was inserted into the SmaI site for in-frame fusion with either DBD alone (pMG) or DBD and PIF1 (pMGPIF1) reading frames. pMG alone was used as a negative control. pT-L and pRNL plasmids have been described (Huq *et al*., 2004). The transient experiments and dual-luciferase assays were carried as described (Huq *et al*., 2004).

***In Vitro* Gel-Shift Assays**

DNA gel shift assays were performed as described (Huq and Quail, 2002). PIF1, PIF1E293D and LUC were synthesized using the Rabbit Reticulocyte TNT system (Promega, Madison, WI). A 70 bp *POR C* promoter fragment containing a G-box motif known to be a PIF1 binding site, was labeled with ^{32}P-dCTP (Su *et al*., 2001; Moon *et al*., 2008). The binding conditions and gel compositions are as described (Huq and Quail, 2002).

RNA isolation and Northern blotting

Total RNA was isolated from 6 day-old seedlings using the Qiagen RNeasy mini kit (Qiagen, Valencia, CA). *RBCS, CAB3 and 18S* cDNA probes (Deng *et al*., 1992) were labeled ([^{32}P]dCTP) using the random primer labeling kit (TaKaRa, Berkeley, CA). A Northern blot was performed on 10 μg total RNA as per manufacturer's instructions using the NorthernMax^{TM}-Gly kit (Ambion, Inc., Austin, TX). After two low stringency and high stringency washes at 42°C, the membrane was dried and exposed to a phosphor screen (Kodak, Rochester, NY) at room temperature overnight. The phosphor screen was developed using the Molecular Imager FX System (Bio-Rad Laboratories, Inc., Hercules, CA).

Accession numbers

Sequence data from this article can be found in the Arabidopsis Genome Initiative under the following accession numbers: *CAB3* (AT1G29910); *PIF1* (AT2G20180); PHYA (AT1G09570); PHYB (AT2G18790); PORC (AT1G03630); *RBCS1A* (AT1G67090); TUBULIN (AT1G04820).

RESULTS

PIF1 Stability Depends on the Quality and Quantity of Light

PIF1 fusion proteins (LUC-PIF1 and PIF1-HA) have been shown to be degraded under both R and FR light conditions (Shen *et al*., 2005; Oh *et al*., 2006). However, the behavior of native PIF1 is unknown. Antibody specific for native PIF1 was raised and used to investigate the stability of native PIF1 under both R and FR light conditions. Native PIF1 was completely degraded in as little as 100 μmolm^{-2} of R light within 2 min. We therefore used 1 μmolm^{-2} of R light to test the degradation kinetics, and the results showed that the half-life of PIF1 was ~1-2 min under these conditions (Figure 2.1A). These results suggested that PIF1 was highly sensitive to R light. Native PIF1 was also degraded under FR light conditions; however, the rate of degradation was much slower

under FR light than under R light (Figure 2.1B). The half-life of native PIF1 was ~5-10 min under 10 μmolm^{-2} of FR light. These results were largely consistent with our previous data using the luciferase fusion protein (LUC-PIF1; Shen *et al*., 2005). However, the small difference in half-life between the native PIF1 and LUC-PIF1 fusion proteins might be due to the overexpression of the fusion proteins using the constitutively active 35S promoter and/or the nature and location of the fusion tags used in previous studies. Overall, these results were consistent with PIF1 having the highest affinity among all the PIFs for the Pfr forms of both phyA and phyB.

phyA Acts Early, While phyB and Other phys Induce PIF1 Degradation under Prolonged Light Conditions

In order to determine the relative contribution of different photoreceptors to light-induced degradation of PIF1, the native PIF1 level in various monogenic and higher order photoreceptor mutant lines was examined. PIF1 was stable in the *phyA* mutant background compared to wild type (wt) after a pulse of FR light (FRp; Figure 2.2A), suggesting that phyA was the sole photoreceptor for PIF1 degradation under FR light. PIF1 was also stable in the *phyA* and *phyAB* backgrounds for up to 60 min after a pulse of R light (Rp; Figure 2.2B). PIF1 degradation was slightly reduced in the *phyB* background compared to wt under these conditions (Figure 2.2B). However, increased light exposure either by greater fluence rates during the light pulse prior to incubation in the dark or prolonged illumination at lower fluence rates showed significant degradation of PIF1 in the *phyA* and *phyAB* backgrounds (Figure 2.2C), suggesting that other phys were involved in PIF1 degradation under these conditions. PIF1 was largely stable in the *phyABD* triple mutant compared to the *phyAB* double mutant background under prolonged R light exposure (Figure 2.2D). These data suggested that phyB, phyD and possibly other phys also contributed to the degradation of PIF1 under prolonged light conditions, presumably when phyA levels were reduced. PIF1 degradation was slightly

reduced in the *cop1* mutant background compared to wt under R light (Figure 2.2B), suggesting that COP1 might play a minor role in regulating PIF1 stability under light.

Light Induces Rapid Phosphorylation, Ubiquitination and Degradation of PIF1

It has been previously shown that PIF1 is degraded through the ubi/26S proteasomal system under R and FR light conditions (Shen *et al*., 2005), but the early events in light-induced degradation of PIF1 have not yet been elucidated. Western blots with anti-PIF1 antibody identified two closely migrating bands under R light condition (Figure 2.3A). These closely migrating bands were absent in dark-grown tissues, and appeared specifically in light-exposed tissues, suggesting that light induced rapid post-translational modification of native PIF1. We investigated whether PIF1 fusion proteins also demonstrated this behavior. Both the LUC-PIF1 and Tandem Affinity Purification (TAP)-PIF1 fusion proteins extracted from the R light-exposed transgenic seedlings migrated as double-bands, suggesting that PIF1 fusion proteins were also post-translationally modified after Rp (Figure 2.3B, C). Both fusion proteins were also degraded following a Rp as expected, while the control proteins (LUC-GFP and TAP-GFP) did not migrate as double-bands, and were not degraded under light (Figure 2.4B; Shen *et al*., 2005), suggesting that the band-shift and the degradation under light were specific to PIF1.

To determine whether the retardation of migration of PIF1 in light-exposed samples was due to a rapid light-induced phosphorylation of PIF1, the immunoprecipitation of TAP-PIF1 fusion protein from dark-incubated, or R or FR light-exposed seedling extracts was performed. The light-exposed samples were then incubated with buffer alone or with active or boiled alkaline phosphatase. The slow migrating PIF1 band in the light-exposed samples was eliminated in the presence of alkaline phosphatase, but not in the presence of boiled (inactive) alkaline phosphatase (Figure 2.3D and E). These results demonstrated that PIF1 was indeed phosphorylated under both R and FR light conditions.

Both LUC-PIF1 and TAP-PIF1 extracted from seedlings that were exposed to a pulse of R light also showed several high molecular weight bands on Western blots (Figure 2.4A, B) that might have been ubiquitinated forms of PIF1. Western blots of immunoprecipitated TAP-PIF1 using anti-ubiquitin (Ubi) antibody showed that TAP-PIF1 recovered from seedlings under either R or FR light conditions was indeed poly-ubiquitinated (Figure 2.4C, D). Anti-MYC (recognizing the the TAP-tag specifically) and anti-Ubi antibodies detected high molecular weight bands from the light-exposed TAP-PIF1 seedling samples, but not in samples immunoprecipitated from seedlings incubated in the dark (Figure 2.4C and D). Additionally, the phosphorylated form of tagged PIF1 predominated in samples immunoprecipitated from the light exposed seedlings (Figure 2.4C, D). These data suggest that PIF1 was phosphorylated and ubiquitinated under both R and FR light conditions before being degraded.

The APB and APA Motifs are Necessary for the Pfr-Specific Interaction of PIF1 with phyA and phyB

To understand the functional significance of PIF1-phy interactions, mapping the phy interaction motifs in PIF1 was carried out. Recent reports showed that a small motif, named the active phytochrome B binding motif (APB), present in many phy-interacting bHLH factors, is necessary for the physical interaction with the Pfr form of phyB (Khanna *et al*., 2004; Al-Sady *et al*., 2006; Shen *et al*., 2007). Alanine scanning by site-directed mutagenesis of conserved amino acids in this region reduced PIF1's interaction with the Pfr form of phyB either severely (E41A, L42A or G47A) or partially (W44A) (Figure 2.5A, B), suggesting that the putative APB motif in PIF1 is also necessary for the interaction with the Pfr form of phyB.

The APB motif of PIF1 was found not to be necessary for interaction with phyA, as a truncated PIF1 (51-478 aa) without the first 50 amino acids interacted with the Pfr form of phyA in a similar manner to full-length PIF1. Al-Sady *et al*. (2006) showed that the two phenylalanine residues (F203 and F209) in PIF3 are necessary for its interaction with phyA. Interestingly, mutations in the corresponding amino acids in PIF1 (F148 and F155) did not disrupt the Pfr-specific binding of PIF1 to phyA. However, deletion of 11 (positions 85 to 95) or 34 (positions 84 to 117) amino acid residues markedly reduced the Pfr-specific interaction of PIF1 with phyA . Deletion of 43 amino acid residues (positions 118 to 160) severely reduced the Pfr-specific interaction of PIF1 to phyA. This region of PIF1 (from residue 84 to residue 160) was scrutinized to identify specific amino acids critical for the PIF1-phyA interaction (data not shown). Site-directed mutagenesis of leucine 95 to alanine showed a similar binding capacity as that of the 11 or 34 amino acid deletion mutants. Site-directed mutagenesis of serine 123, glycine 153 and glycine 160 to alanines in the leucine 95 mutant background did not show significant differences in binding compared to the leucine 95 single mutant (data not shown). However, site-directed mutagenesis of asparagine 144 to alanine in the leucine 95 mutant background showed that these two amino acid residues were necessary for the interaction with the Pfr form of phyA *in vitro* (Figure 2.5C). These results suggested that the phyA binding sites were different between PIF1 and PIF3.

Experiments involving *in vitro* transcribed and translated PIFs and phys, have shown that interactions among them occur, but neither *in vivo* interactions nor interactions between plant-expressed proteins have been demonstrated. To investigate whether PIF1 interacts with phyA or phyB *in vivo* and to examine the involvement of specific amino acids in PIF1-phy interactions *in vivo*, we generated homozygous transgenic plants expressing LUC-PIF1-3M (a luciferase-PIF1 fusion protein with three mutations in PIF1: G47A, L95A and N144A) in the *pif1* mutant background. We performed co-immunoprecipitation assays using the anti-PIF1 antibody on samples prepared from dark and light-exposed plants. Results showed that LUC-PIF1 could efficiently interact with

both phyA and phyB from plant extracts (Figure 2.5D). However, co-immunoprecipitations of LUC-PIF1-3M recovered much less phyA and phyB under R light compared to LUC-PIF1 co-immunoprecipitations (Figure 2.5D). These results were consistent with the *in vitro* interactions shown in Figure 2.5B and C. Taken together, these data suggested that the three amino acids (G47, L95 and N144) in PIF1 were critical for physical interactions with the Pfr forms of phyA and phyB both *in vitro* and *in vivo*.

The Light-Induced Phosphorylation and Degradation of PIF1 Requires the Direct Interaction with the Pfr Forms of either phyA or phyB

In order to determine if direct physical interactions with phys are necessary for the degradation of PIF1 in light, we generated homozygous transgenic plants expressing LUC-PIF1G47A or LUC-PIF1-2M (containing two mutations in PIF1: L95A and N144A) in the *pif1* background. LUC-PIF1-3M (containing three mutations in PIF1: G47A, L95A and N144A) is described above. Luciferase assays showed that the degradation of the LUC-PIF1G47A (deficient in interaction with phyB) was slightly reduced under prolonged R light (data not shown). The triple mutant LUC-PIF1-3M (deficient in interaction with phyA and with phyB) was completely stable under FR light and only partially degraded under prolonged R light (data not shown). To investigate the early kinetics of degradation, we performed cycloheximide (CHX) chase assays for the wt and the mutant forms of PIF1 fused to LUC after a pulse of R light followed by dark incubation (Figure 2.6A). The degradation rate of LUC-PIF1G47A was similar to the wt LUC-PIF1 under these conditions (Figure 2.6B), suggesting that phyB plays a minor role in early PIF1 degradation under limited R light. However, the degradation rates of both LUC-PIF1-2M and LUC-PIF1-3M were greatly reduced after a pulse of R light compared to those of LUC-PIF1 (Figure 2.6C). Moreover, LUC-PIF1-3M was neither phosphorylated nor degraded up to 20 min after a pulse of R light, whereas wt LUC-PIF1 was both phosphorylated and degraded under these conditions (Figure 2.6D). These

results, and those depicted in Figure 2.5D, suggested that direct interactions of PIF1 with phys were necessary for the light-induced phosphorylation and degradation of PIF1.

The Transcriptional Activation Domain and phy-Interaction Domain are Both Present in the N-Terminal 150 Amino Acid Region of PIF1

Published research in other systems besides plants, have shown that the protein degradation domains can overlap with transcription activation domains (Salghetti *et al*., 2000; Muratani and Tansey, 2003). To investigate whether the degradation domain and transcription activation domain of PIF1 overlap, we mapped the transcription activation domain of PIF1 using the transient assay system we developed (Huq *et al*., 2004). The N-terminal 150 amino acid region had the transcriptional activation activity of PIF1 (Figure 2.7). Strikingly, the transcription activation domain overlapped with the APB and APA motifs of PIF1 (Figure 2.5) that were necessary for PIF1 interaction with photoactivated phys and subsequent degradation in light.

The Light-Induced Degradation of PIF1 Requires Both N- and C-Terminal Domains of PIF1

In order to map the degradation domain of PIF1 translational fusions of LUC with one of two regions of PIF1 were generated. The selected regions of PIF1 were amino acid residues 1-150, responsible for phy interaction and including the transcriptional activation domain of PIF1, and residues 151-478, responsible for dimerization and DNA binding) as described (Shen *et al*., 2005), and produced transgenic plants. To examine whether dimerization was necessary for PIF1 degradation, we also produced transgenic plants expressing LUC fused to the 1-150 amino acid region of PIF1 along with the bHLH domain (Figure 2.8A). We measured LUC activity as an indicator of fusion protein stability under dark and light conditions as described (Shen *et al*., 2005). All three truncated fusion proteins were stable under both R and FR light, while the full-length

LUC-PIF1 fusion protein was degraded under those conditions as expected (Figure 2.8B). Western blot analyses of two of the truncated proteins (LUC-PIF1-N150 and LUC-PIF1-C327) showed that these fusion proteins were neither phosphorylated nor degraded under R light (Figure 2.8C). These results strongly suggested that both the N- and C-terminal regions of PIF1 were necessary, but not sufficient, for the light-induced degradation of PIF1. In addition, since the phy-interaction motifs were present in the 1-150 amino acid region of PIF1, these results together with the above point mutations (Figures 2.5, 2.6) suggest that phy binding was necessary, but not sufficient, for PIF1's light-induced degradation. Moreover, the transcriptional activation domain of PIF1 was necessary, but not sufficient, to orchestrate light-induced PIF1 degradation.

The Light-Induced Degradation of PIF1 does not Require DNA Binding

It has been shown in other systems besides plants that transcription factors are often tagged for subsequent degradation by the ubi/26S proteasomal pathway while they are assembled in the transcription initiation complex bound to their DNA target (Mayr and Montminy, 2001; Muratani and Tansey, 2003). Davis *et al*. (1990) showed that a single amino acid substitution (E118D) in MYOD, a bHLH protein, abolished its DNA binding activity. To investigate whether DNA binding was necessary for the light-induced degradation of PIF1, we introduced the above missense mutation in the corresponding amino acid of PIF1 (PIF1E293D), and compared the DNA binding activity of the wt and mutant PIF1. The mutant PIF1 did not bind to the target DNA while the wt PIF1 showed robust binding (Figure 2.9A and B). We made a LUC-PIF1E293D fusion construct and generated homozygous transgenic plants expressing the fusion protein in the *pif1* mutant background. LUC assays showed that this mutant PIF1 (PIF1E293D) was degraded significantly more than the wild type.

Overexpression of the Light-Stable Truncated Form of PIF1 Induces Constitutive Photomorphogenic Phenotypes in the Dark

In order to determine the biological functions of the various missense and truncated PIF1 mutants, homozygous lines for plants expressing LUC-PIF1-3M, LUC-PIF1-E293D, LUC-PIF1-N150 and LUC-PIF1-C327 were selected. The seedling phenotypes were compared with those of LUC-PIF1 transgenic lines. As previously shown, none of these transgenic plants complemented the seed germination phenotypes of the *pif1* mutant, possibly due to the use of the 35S promoter (Shen *et al*., 2005). However, both LUC-PIF1 and LUC-PIF1-3M complemented the seedling phenotypes of the *pif1* mutant, including hypocotyl lengths, chlorophyll content, bleaching phenotypes and hypocotyl negative gravitropism to a similar extent. LUC-PIF1-E293D showed increased levels of chlorophyll content and shorter hypocotyls compared to the *pif1* mutant. These data suggested that the LUC-PIF1-E293D fusion protein not only failed to complement the *pif1* phenotypes, but also displayed enhanced hypersensitive phenotypes compared to the *pif1* mutant, possibly due to dominant-negative effects. LUC-PIF1-N150 did not complement any of the above phenotypes (data not shown). Strikingly, LUC-PIF1-C327 induced a constitutively photomorphogenic phenotype in the dark in a dose-dependent manner (Figure 2.10A-F). These seedlings had open and expanded cotyledons, and showed shorter hypocotyls and increased levels of photosynthetic gene expression compared to those of the wt seedlings in the dark. Moreover, these seedlings also showed greener and larger (more expanded) cotyledons, and shorter hypocotyls compared to wt seedlings when grown under light (Figure 2.11). It is possible that the truncated form of PIF1 is functioning in a dominant negative manner to induce constitutive photomorphgenic phenotypes in the dark.

DISCUSSION

Since PIFs are able to physically interact with the photoactivated phytochrome molecules, PIFs were thought to receive light signals from phys and induce photomorphogenesis (Ni *et al*., 1998, 1999; Quail, 2002). However, contrary to our expectation, the majority of the biological functions of the PIF family members are to negatively regulate phy signaling (Castillon *et al*., 2007; Monte *et al*., 2007). To remove this negative regulation, phys induce degradation of PIFs in order to promote photomorphogenesis. Here we present evidence that, using diverse sequences, phys interact with PIF1 to induce its phosphorylation, poly-ubiquitination and subsequent degradation under both R and FR light conditions. Moreover, overexpression of a light-stable truncated form of PIF1 induced constitutively photomorphogenic phenotypes in the dark (Figure 2.10), suggesting that an inactivation of PIFs by higher-order mutation might be sufficient to induce photomorphogenesis constitutively in the dark.

These results show that PIF1, the member with the highest affinity for phys, is highly sensitive to the quality and quantity of light. The half-life of native PIF1 was ~1-2 min under 1 $\mu molm^{-2}$ of R light (Figure 2.1). Other PIFs, including PIF3, PIF4 and PIF5 are degraded with varying but lower sensitivity under R and/or FR light conditions (Bauer *et al*., 2004; Monte *et al*., 2004; Lorrain *et al*., 2007; Nozue *et al*., 2007; Shen *et al*., 2007; Leivar *et al*., 2008). phyA is also degraded under R light through the ubi/26S proteasomal pathway (Shanklin *et al*., 1987). However, native PIF1 is much more sensitive to R light compared to all other known light-labile proteins. Because of phyA's high sensitivity and early role in responding to light signals, it played the dominant role in regulating the stability of PIF1 under low R light intensity (2 $\mu molm^{-2}$) (Figure 2.2A). However, under high R light intensity (3000 $\mu molm^{-2}$), phyB, phyD and possibly other phys also influenced PIF1 stability. The native PIF1 was also significantly more sensitive to light than the PIF1 fusion proteins originally used to demonstrate light-induced degradation of PIF1 (Shen *et al*., 2005; Oh *et al*., 2006; Castillon *et al*., 2007; Quail, 2007). The small

difference in degradation rate might be due to the overexpression of the fusion proteins using the constitutively active 35S promoter and/or to the differential affinities for the wt and PIF1 fusion proteins toward phys. Taken together, these results now demonstrate that PIF1 is one of the most light-sensitive proteins known in plants, which is consistent with PIF1 having the strongest affinity for both phyA and phyB among all the PIFs (Huq *et al*., 2004). The strong light-sensitivity of PIF1 is also consistent with its role in regulating seed germination. In natural conditions, seeds buried under soil are exposed to a small amount of light penetrating through the soil surface, and that might be sufficient to degrade PIF1 to allow the completion of germination (Oh *et al*., 2004; Oh *et al*., 2007).

The data presented here demonstrate that PIF1 was phosphorylated and poly-ubiquitinated specifically under both R and FR light conditions before being degraded by the proteasomal pathway (Figures 2.3, 2.4). Light-induced phosphorylation and poly-ubiquitination of PIF3 and PIF5 have recently been shown (Park *et al*., 2004; Al-Sady *et al*., 2006; Lorrain *et al*., 2007; Shen *et al*., 2007). However, these alterations were seen only under R light. Both PIF3 and PIF5 are also degraded under FR light, but the early steps in FR-induced degradation are not yet known. Our results suggest that the early events in both R and FR-induced degradation of PIFs might be their phosphorylation and polyubiquitination followed by degradation by the ubi/26S proteasomal pathway.

PIF family members can interact selectively with the Pfr form of phys *in vitro* (Ni *et al*., 1999; Huq *et al*., 2004). Sequence alignment and site-directed mutagenesis revealed that an N-terminal motif, named the active phyB binding motif (APB), is necessary for the physical interactions between PIFs 3-7 and phyB *in vitro* (Khanna *et al*., 2004; Shen *et al*., 2007; Leivar *et al*., 2008). A second motif immediately downstream of the APB motif, named the active phyA binding motif (APA) (data not shown), has been shown to mediate interactions between PIF3 and phyA (Al-Sady *et al*., 2006). Here we show that while PIF1 had a functionally conserved APB motif (Figure 2.5), it used a novel APA

motif for interaction with the Pfr form of phyA (Figure 2.5C). The APA and APB motifs were necessary for the robust interaction with phyA and phyB, respectively both *in vitro* and *in vivo* (Figure 2.5C, D). Moreover, because the triple mutant still interacted with phyA/phyB *in vivo*, perhaps additional amino acid residues in PIF1 participate in physical interactions between PIF1 and phys *in vivo*. Combined, these data suggest that although phyB uses a highly conserved sequence motif for physical interactions with PIFs, phyA uses a more diverse sequence for physical interactions with PIFs. Identification and functional characterization of additional phyA interacting factors might reveal whether phyA uses any conserved sequence motif for physical interaction.

The functional significance of PIF-phy physical interactions appears antagonistic. Direct interactions with phys are necessary for the light-induced phosphorylation and degradation of PIF1, because a PIF1 triple mutant deficient in phy interaction displayed reduced levels of phosphorylation and degradation under light (Figure 2.6). These results are consistent with the recent reports that physical interactions with phys are necessary for the light-induced phosphorylation and degradation of PIF3/PIF5 (Al-Sady *et al*., 2006; Lorrain *et al*., 2007; Shen *et al*., 2007). However, expression of two separate regions of PIF1 (1-150 aa containing the transcriptional activation domain as well as the APA and APB motifs, and 151-478 aa containing the dimerization domain) in transgenic plants showed that these isolated regions were neither phosphorylated nor degraded under either R or FR light conditions (Figure 2.8). Because the phy-interaction motifs are present at the N-terminal 150 amino acid region of PIF1 (Figure 2.5), these results demonstrate that although the physical interactions between PIF1 and phys are necessary, they are not sufficient for the light-induced phosphorylation and degradation of PIF1. Therefore, PIFs might have additional molecular determinants for light-induced phosphorylation and degradation. Further characterizations of amino acid residues using site-directed mutagenesis are necessary to identify these regions.

These experiments demonstrate that the putative transcription activation domain of PIF1 was necessary, but not sufficient for its light-induced degradation (Figure 2.7) suggest that not all transcription activation domains function as "degrons" as previously hypothesized (Salghetti *et al*., 2000; Muratani and Tansey, 2003). Moreover, enhanced degradation of the PIF1 mutant that failed to bind to DNA also suggests that DNA binding may inhibit PIF1 degradation (Figure 2.9). These results are consistent with previous reports that a small fraction of PIF1 (20-30%) was not degraded even under continuous light exposure (Shen *et al*., 2005). Taken together, these results suggest that the light-induced degradation of PIF1 might be nucleoplasmic and is uncoupled from the transcription complex.

Although, the interaction motifs in PIFs have been the focus of recent investigations, the interaction motifs in phys have not been investigated in detail. PIF3 has been shown to interact with the N- and C-terminal halves of phyB separately (Ni *et al*., 1999; Zhu *et al*., 2000). Moreover, PIF3 showed higher affinity for the Pfr form of the N-terminal half compared to the nonphotoactive C-terminal half of phyB. The Pfr form of full-length phyB showed a greater and synergistic affinity for PIF3 relative to the two isolated halves. Sequence regions of phyA for PIF3 interaction are not yet known. Moreover, neither phyA nor phyB interact with the DNA-bound PIF1 *in vitro* (Huq *et al*., 2004). Interactions between PIF3 and the N- or C-terminal halves of phyB have been interpreted to explain the biological functions of the N-terminal half of phyB in transgenic plants (Ni *et al*., 1999; Zhu *et al*., 2000; Matsushita *et al*., 2003; Oka *et al*., 2004). Our data suggest that phy signaling through the direct interaction of the N-terminal half of phyB with PIF3 may not represent a general mechanism for all the PIFs as previously proposed.

Experiments which involved *pif* monogenic mutants did not reveal any significant role of PIFs in regulating the morphological phenotypes of dark-grown seedlings. However, the hypersensitive phenotypes of *pif3*, *pif4*, *pif5* single and higher-order

mutants under prolonged R light have recently been shown to be due to an increased level of phyB (Monte *et al*., 2004; Leivar *et al*., 2008). By contrast, the chlorophyll biosynthetic and seed germination defects of the *pif1* mutant are due to a mis-regulation of these pathways in the dark (Huq *et al*., 2004; Oh *et al*., 2006; Oh *et al*., 2007). PIF1 directly and indirectly regulates the key genes in the chlorophyll biosynthetic pathway in the dark to optimize the greening process in *Arabidopsis* (Moon *et al*., 2008). Moreover, both PIF1 and PIF3 constitutively activate transcription in the dark, which is reduced under light presumably due to their light-induced degradation. (Bauer *et al*., 2004; Huq *et al*., 2004; Shen *et al*., 2005; Al-Sady *et al*., 2008). Consistent with these results, it is striking that the overexpression of a light-stable truncated form of PIF1 induced constitutively photomorphogenic phenotypes in the dark (Figure 2.10). These transgenic plants showed both morphological and molecular phenotypes qualitatively similar to the *constitutive photomorphogenic 1* (*cop1)* mutant in a dose-dependent manner. This region of PIF1 contains the bHLH dimerization and DNA binding domains without the transcriptional activation domain (Figure 2.7). It is possible that this region is functioning in a dominant negative manner by heterodimerizing with other PIFs and titrating out their activity in the dark. These data suggest that simultaneous removal of all PIFs by light-mediated degradation might be sufficient to induce photomorphogenesis. Alternatively, photomorphogenesis might be induced in the dark by overexpression of a dominant-negative form of PIF or possibly by creating a higher-order mutant of *PIFs*. This hypothesis is consistent with a recent report that overexpression of constitutively photoactive phyA and phyB induces photomorphogenesis in the dark (Su and Lagarias, 2007), presumably due to light-independent degradation of PIFs in the dark. Taken together, these results suggest that PIFs negatively regulate photomorphogenesis not only in the light, but also in the dark.

In conclusion, PIF1 and possibly other PIFs appear to play major roles in the dark to inhibit photomorphogenesis (Figure 2.12, left). Light-activated photoreceptors directly interact with PIFs to induce their phosphorylation, poly-ubiquitination and subsequent

degradation via the ubi/26S proteasomal pathway in order to promote photomorphogenesis (Figure 2.12, right). Because direct physical interactions of PIFs with phys are necessary for the light-induced phosphorylation of PIFs, and because phyA has been shown to function as a non-conventional serine/threonine kinase (Yeh and Lagarias, 1998), it is possible that phys can directly phosphorylate PIFs. However, convincing *in vivo* evidence of phyA kinase activity is still lacking. Therefore, it remains to be determined whether the light-induced phosphorylation of PIFs represents the primary biochemical mechanism of phy signal transfer or whether phys simply function as scaffold proteins to bring the PIFs and another unknown kinase together for the phosphorylation event.

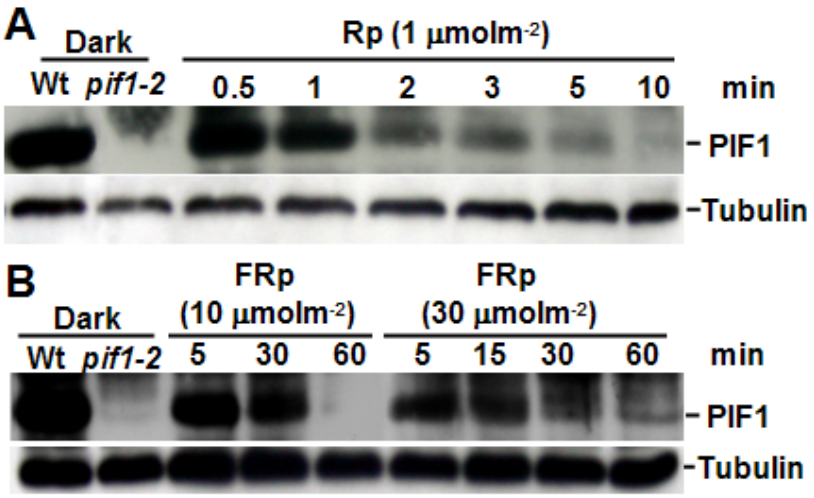


Figure 2.1: PIF1 stability is highly sensitive to the quality and quantity of light. Native PIF1 is rapidly degraded under pulses of red (Rp) (1 μmolm^{-2}) (A) or far-red (FRp) (10 or 30 μmolm^{-2}) (B) light conditions. Four day-old dark-grown seedlings were exposed to Rp or FRp light and then incubated in the dark for the durations indicated before being harvested for protein extraction. Protein extracts from dark-grown wild type and *pif1* null mutant are also included in the first two lanes, respectively. Approximately 30 μg of total protein in each lane was separated on an 8% polyacrylamide gel, transferred to PVDF membrane and probed with anti-PIF1 antibody. A similar blot was probed with anti-tubulin antibody. The bands corresponding to PIF1 and tubulin are labeled.

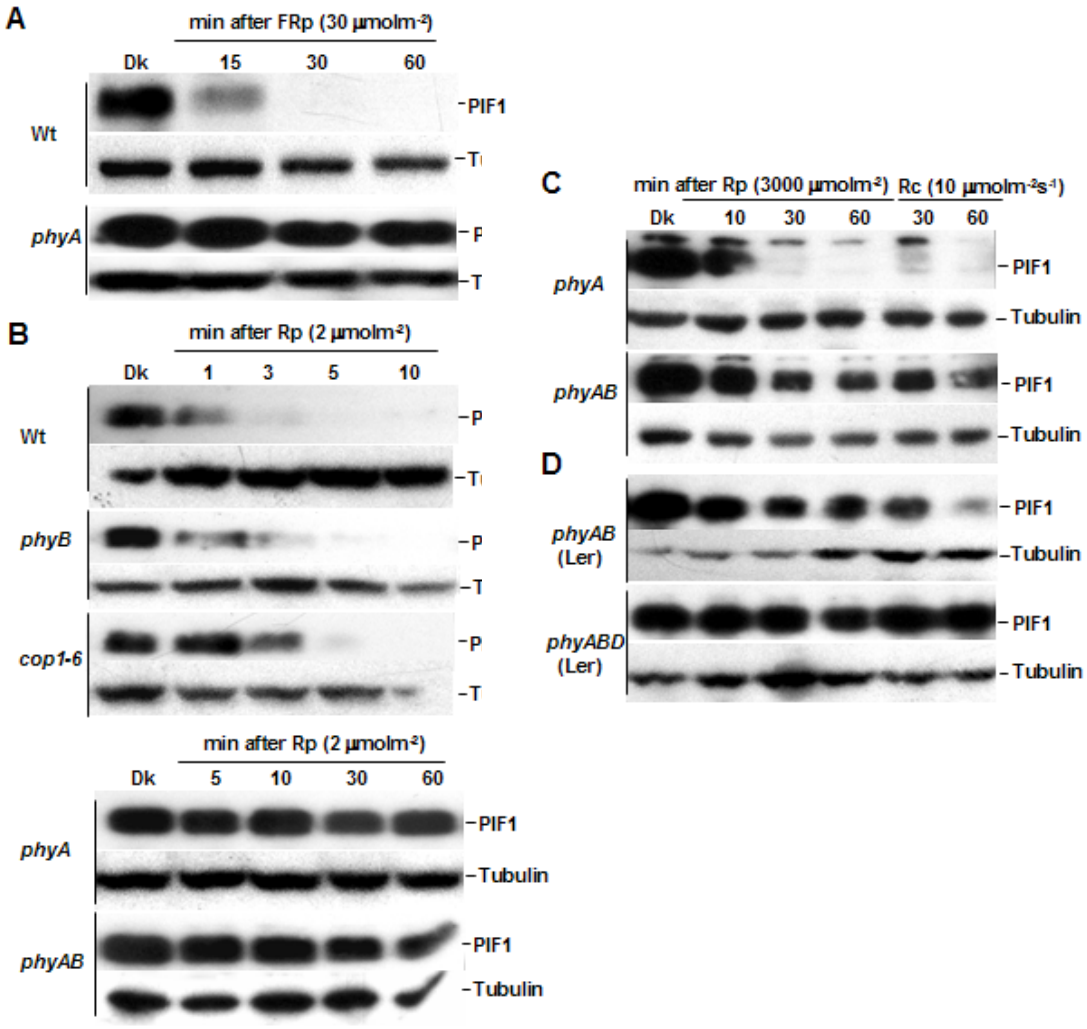


Figure 2.2: phyA plays a dominant role during the initial light exposure while phyB, phyD and other phys regulate PIF1 stability under prolonged light exposure. Western blots showing native PIF1 levels in wt, *phyA*, *phyB* and *phyAB* double mutant backgrounds. Four day-old dark-grown seedlings were exposed to a pulse of far-red light (FRp; A), a pulse of red light (Rp; B, C and D) or continuous red light (Rc; C and D) at the indicated fluences, and then incubated in the dark for the time indicated (except Rc) before harvesting for protein extraction. *phyAB* and *phyABD* shown in D are in Ler ecotype, while all other mutants are in Col-O ecotype.

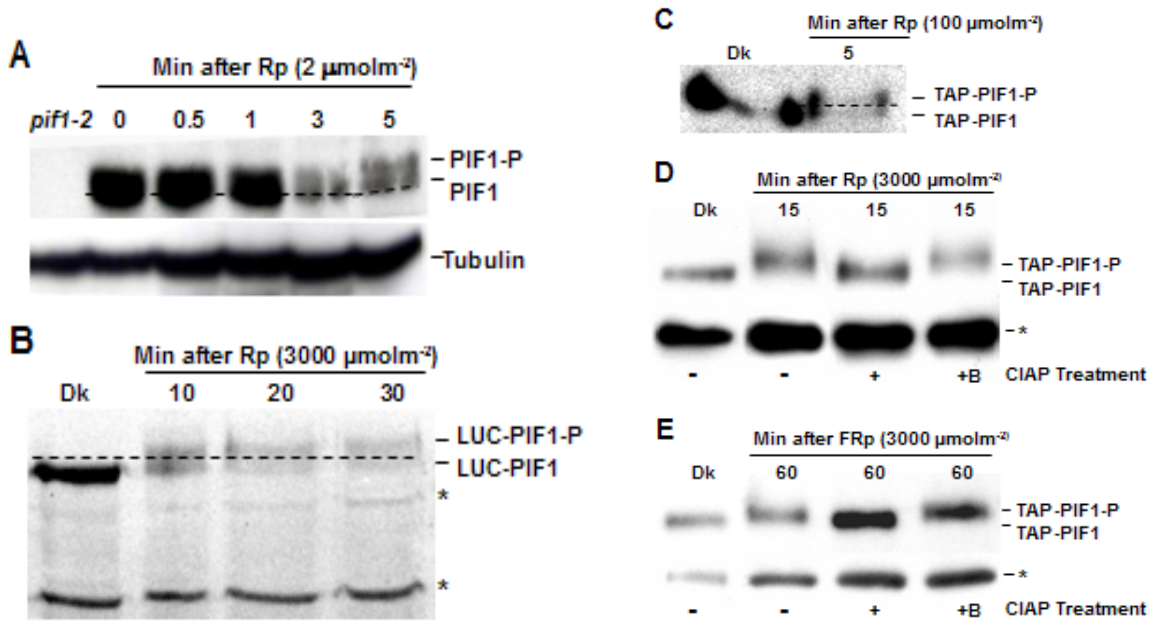


Figure 2.3: Light induces rapid phosphorylation prior to degradation of PIF1. A) Native PIF1 migrates as two bands (PIF1 and PIF1-P) following a pulse of red light (Rp; 2 µmolm^{-2}). A blot probed with anti-PIF1 antibody is shown. B) LUC-PIF1 also exhibits a slower migrating band (LUC-PIF1-P) after a Rp (3000 µmolm^{-2}). Proteins from plants expressing LUC-PIF1 were probed with anti-LUC antibody. C) TAP-PIF1 shows a slower migrating band (TAP-PIF1-P) and is also degraded after a Rp (100 µmolm^{-2}). Proteins from plants expressing TAP-PIF1 were probed with anti-MYC antibody which recognizes the TAP tag. Dotted lines separate the two forms of PIF1 in A-C. D and E) The Rp- and FRp-induced slow migrating band is a phosphorylated form of PIF1. TAP-PIF1 was immunoprecipitated from protein extracts prepared using four day-old dark-grown 35S:TAP-PIF1 seedlings kept in the dark or exposed to either Rp (3000 µmolm^{-2}; D) or FRp (3000 µmolm^{-2}; E) followed by dark incubation. The immunoprecipitated pellets from the Rp- or FRp-exposed samples were dissolved in buffer and incubated without (-) or with (+) native Calf Intestine Alkaline Phosphatase (CIAP) or with boiled CIAP (+B). Samples were then separated on 6.5% SDS-PAGE gels and probed with anti-MYC antibody. Asterisks denote cross-reacting bands.

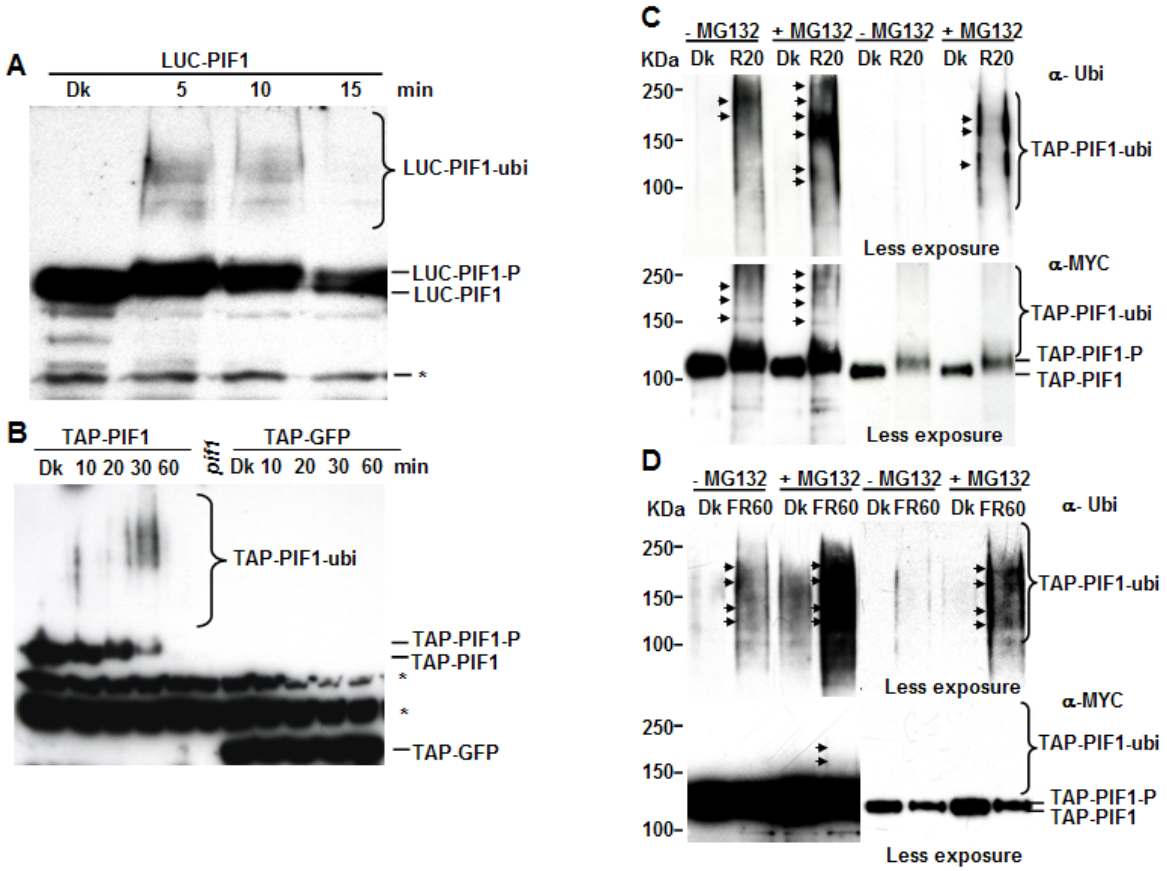


Figure 2.4: Light induces rapid phosphorylation and ubiquitination prior to degradation of PIF1. A) LUC-PIF1 shows high molecular weight bands (LUC-PIF1-ubi) after a Rp (3000 μmolm^{-2}). A blot probed with anti-LUC antibody is shown. B) TAP-PIF1 shows high molecular weight bands (TAP-PIF1-ubi) and is also degraded following a Rp (100 μmolm^{-2}), while TAP-GFP is stable under these conditions. A blot probed with anti-MYC antibody which recognizes the TAP tag is shown. Asterisks denote cross-reacting bands. C and D) The Rp- and FRp-induced slow migrating bands are ubiquitinated forms of PIF1. TAP-PIF1 was immunoprecipitated from protein extracts prepared using four day-old dark-grown seedlings either kept in the dark (Dk) or exposed briefly to Rp light (3000 μmolm^{-2}; C) or FRp light (3000 μmolm^{-2}; D). The immunoprecipitated samples were then separated on 6.5% SDS-PAGE gels and probed with anti-ubiquitin (Ubi) or anti-MYC antibodies. Data obtained by Hui Shen.

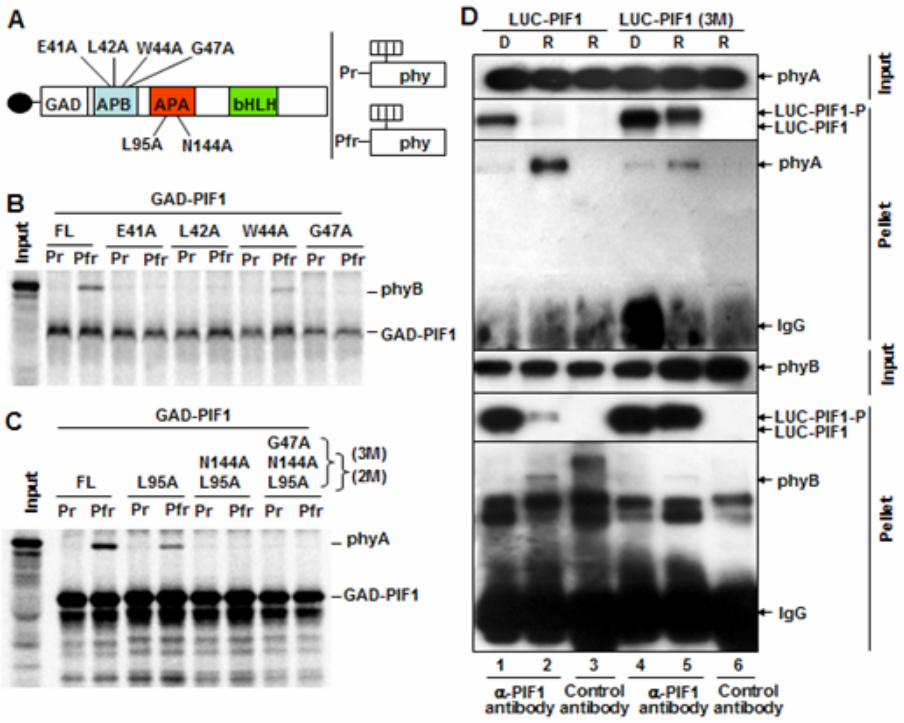


Figure 2.5: The APB and APA motif present in the N-terminal 150 amino acid region of PIF1 is necessary for its Pfr-specific interaction with phyA and phyB both *in vitro* and *in vivo*. A) Schematic representation of the gal4 activation domain-PIF1 (GAD-PIF1) baits (left) and full-length phy (phy) preys (right) used in co-immunoprecipitation assays. Mutations made in GAD-PIF1 for testing phyB binding are shown above the schematic, and those for testing phyA binding are below. Autoradiographs show *in vitro* interactions of wild type PIF1 or each of four PIF1 mutants with the Pr or Pfr forms of phyB (B) or single, double (2M) or triple (3M) mutants of PIF1 with the Pr or Pfr forms of phyA (C). The leftmost lane of each panel shows the input and and the others show the pellet fractions from co-immunoprecipitation assays performed with *in vitro* synthesized bait and prey proteins. The phyA and phyB holoproteins were reconstituted by adding the chromophore. The baits were immunoprecipitated using anti-GAD antibody. D) LUC-PIF1-3M shows much less affinity for the Pfr forms of phyA and phyB compared to LUC-PIF1 in *in vivo* co-imunoprecipitation assays. The input and pellet fractions from *in vivo* co-immunoprecipitation assays are indicated. Total protein was extracted from four day-old dark-grown seedlings either exposed to Rp light (R; 3000 $\mu molm^{-2}$) or kept in the dark (D). Co-immunoprecipitations were carried out using the anti-PIF1 antibody (lanes 1, 2, 4 and 5) or with and unrelated IgG as a control (lanes 3 and 6). The immunoprecipitated samples were then probed with anti-phyA, anti-phyB or anti-LUC antibodies. Data obtained by Hui Shen and Ling Zhu.

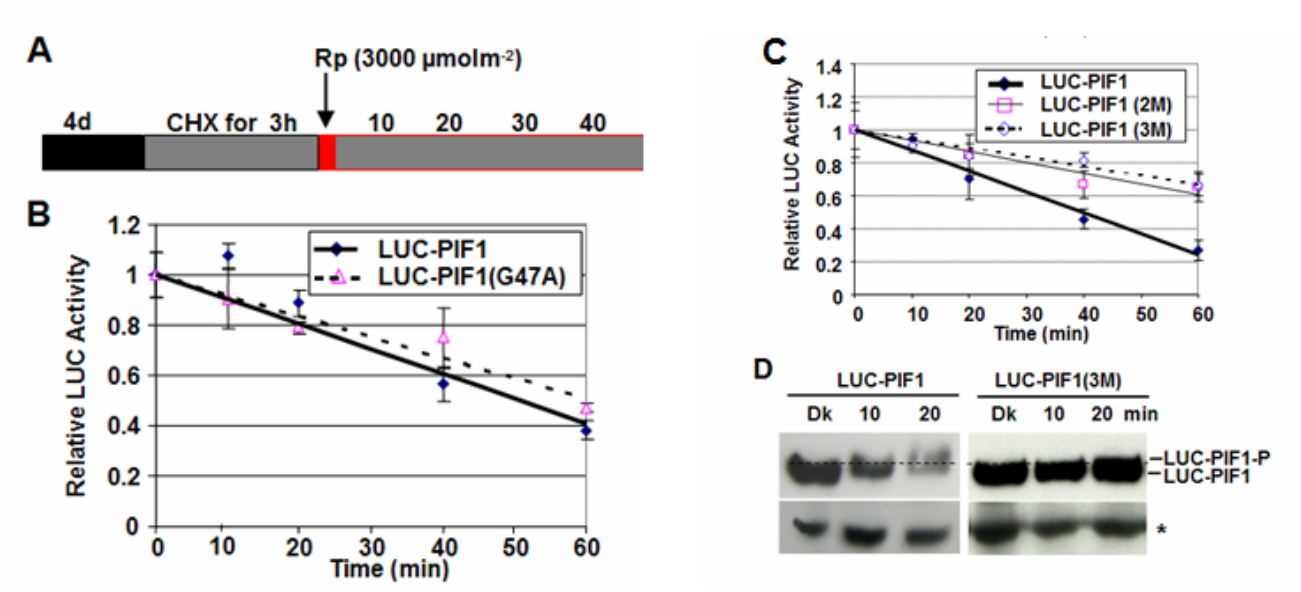


Figure 2.6: Interactions with the Pfr form of phyA and phyB are necessary for the light-induced phosphorylation and degradation of PIF1. A) Design of the cycloheximide chase assays. Relative lucerifase activity for phy-interaction deficient mutants was measured in 4-day-old (4d) dark-grown seedlings pretreated with cycloheximide (CHX) in the dark for 3h, exposed to R (3000 µmolm^{-2}) light and then incubated in the dark for the indicated time (min). Assays show the kinetics of degradation of LUC-PIF1-G47A (B) and LUC-PIF1-2M and LUC-PIF1-3M (C) compared to wt LUC-PIF1. LUC-PIF1G47A is deficient in phyB interaction, LUC-PIF1-2M is deficient in phyA interaction and LUC-PIF1-3M is deficient in both phyA and phyB interaction as shown in Figure 5. Means ± SE of five biological replicates are shown. (D) The abundance and phosphorylation status of LUC-PIF1 or LUC-PIF1-3M fusion proteins prior to and after exposure to a Rp determined in Western blots using anti-LUC antibody. A dotted line separates the two forms of PIF1. The asterisk denotes a cross-reacting band. Data obtained by Hui Shen and Ling Zhu.

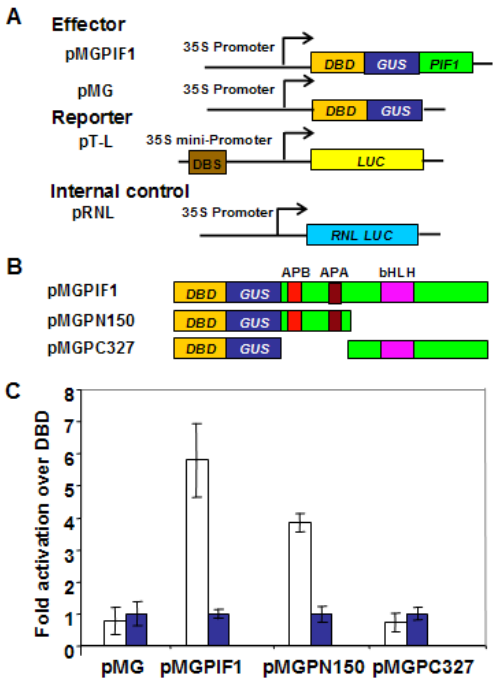


Figure 2.7: Transcriptional activation domains are located at the N-terminus of PIF1. (**A**) Constructs used for the experiment. The effector constructs were designed to express a GAL4 DNA binding domain (DBD) PIF1 fusion (pMGPIF1) or the GAL4 DNA binding domain alone (pMG). The reporter construct (pT-L) expresses a firefly luciferase (LUC) from the 35S minimal promoter fused to gal4 DNA binding site (DBS). The internal control (pRNL) expresses a renilla luciferase (RNL LUC) from the 35S promoter. (B) PIF1 deletion constructs used to map the transcriptional activation domains. Each effector construct in A and B is fused to β-glucuronidase (GUS) to permit the determination of the expression level of the fusion proteins. (C) Three-day-old etiolated *Arabidopsis* seedlings were co-bombarded with the reporter and effector constructs. Seedlings were treated for 15 min with far-red light and then incubated in darkness for 16 h. Means ± SE from four biological replicates are shown. Transcriptional activity was measured in seedling extracts by a dual-luciferase assay system (Promega, Madison, WI). Fold-activation is expressed as transcriptional activation activity of DBD-GUS-PIF1 over transcriptional activity of DBD-GUS (white bar) and normalized with GUS activity for the amount of protein expressed by each construct (blue bar).

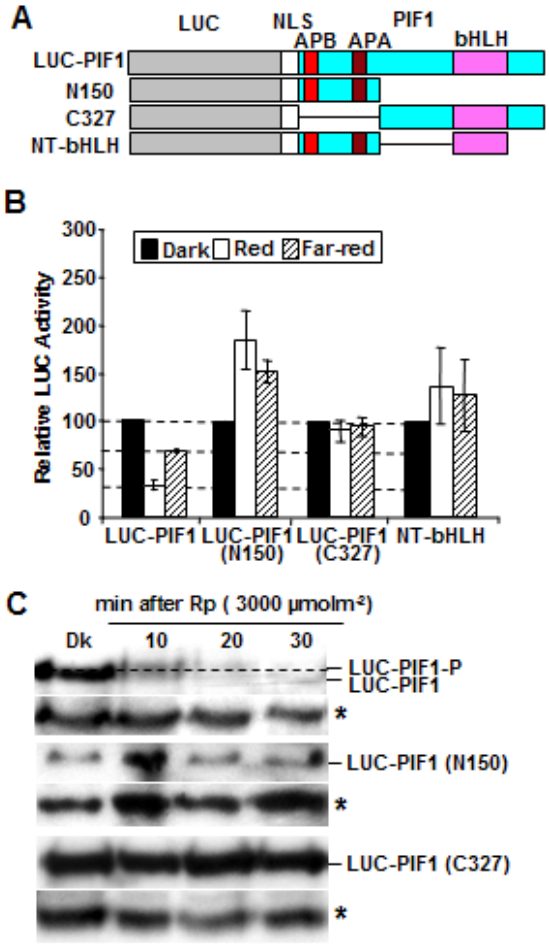


Figure 2.8: Both the N- and C-terminus of PIF1 are necessary for the light-induced degradation of PIF1. A) Design of the PIF1 deletion constructs fused to LUC. The white box represents a nuclear localization signal (NLS). B) LUC activity was measured from 4-day-old dark-grown seedlings transferred to R (10 µmolm^{-2}s^{-1}) or FR (10 µmolm^{-2}s^{-1}) light for 1 h as described (Shen *et al*., 2005). Means ± SE of five biological replicates are shown. Some constructs showed greater stability of the fusion protein in light relative to darkness for unknown reasons. C) Western blots showing truncated PIF1 fusion proteins are neither phosphorylated nor degraded under light, but the wt LUC-PIF1 is both phosphorylated and degraded under light. A dotted line separates the two forms of PIF1. Asterisks denote a cross-reacting band. Data obtained by Hui Shen and Ling Zhu.

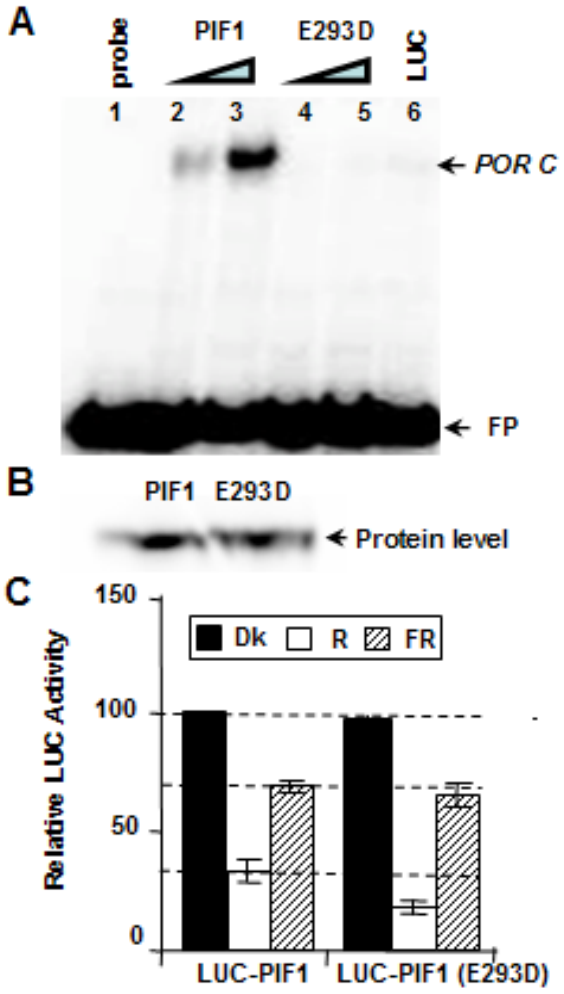


Figure 2.9: DNA binding is not necessary for the light-induced degradation of PIF1. A) The PIF1E293D mutant does not bind to a G-box DNA sequence element (*POR C*; Su *et al*., 2001; Moon *et al*., 2008). *In vitro* translated PIF1 or PIF1E293D was incubated with a radiolabeled fragment of *POR C* in a DNA gel shift assay. Lane 1, free probe; lanes 2-3, increasing amount of wt PIF1; lanes 4-5, increasing amount of PIF1E293D mutant protein, and lane 6, unrelated luciferase protein as a negative control. FP, free probes. B) Comparison of the levels of wt and mutant PIF1 proteins produced by *in vitro* transcription and translation. C) Relative LUC assays were performed under conditions described in Figure 8. Means ± SE of five biological replicates are shown. Data obtained by Hui Shen and Ling Zhu.

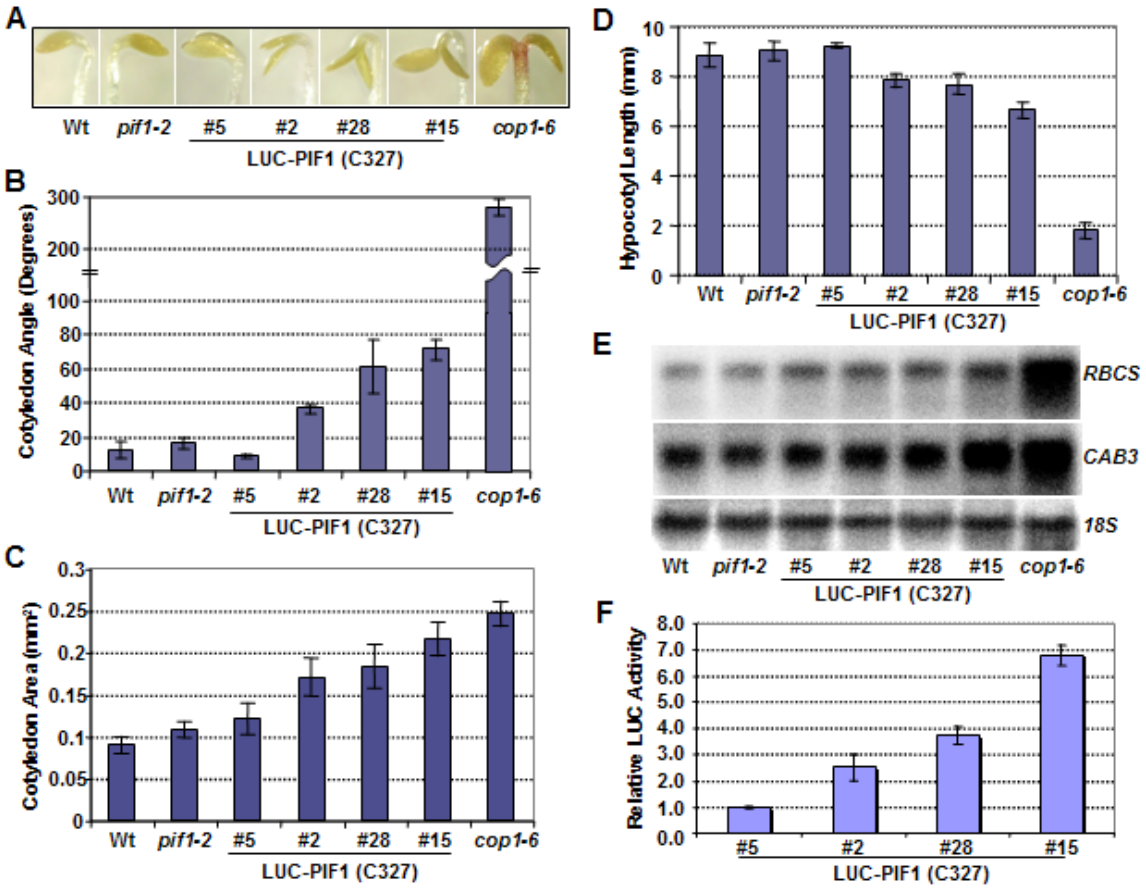


Figure 2.10: Overexpression of the light-stable, truncated form of PIF1 (C327) induces a constitutive photomorphogenic phenotype in the dark. Visible cotyledon opening phenotypes of various lines grown in the dark for 4 days (A). Measurement of cotyledon angles (B), cotyledon areas (C) and hypocotyl lengths (D) of various lines grown in the dark for 4 days (means ± SE; n≥30). E) Photosynthetic gene expression is higher in the C327 lines compared to wt in the dark. RNA was extracted from 4 day-old dark-grown seedlings and probed for the indicated photosynthetic (RBCS and CAB3) or non-photosynthetic control (18S) transcripts. F) Luciferase activity of various LUC fusion proteins as an indicator of C327 protein amounts in the independent transgenic lines. Relative LUC assays were performed from 4 day-old dark-grown seedlings as described (Shen *et al*., 2005).

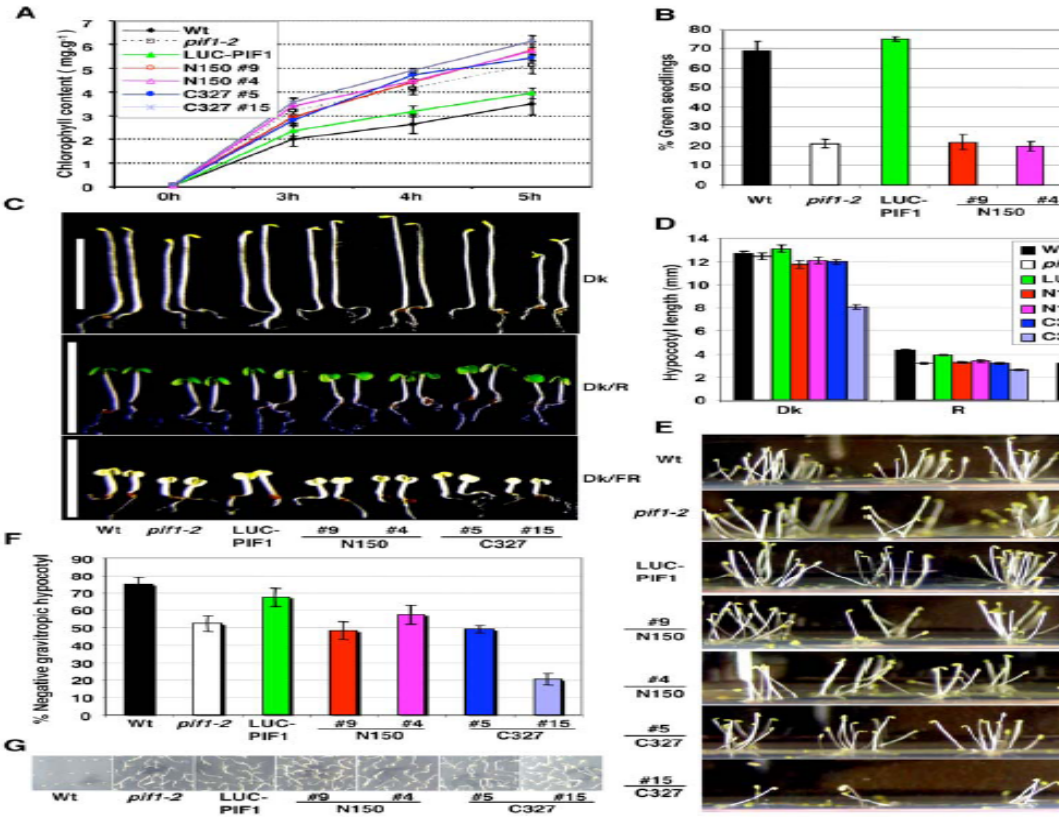


Figure 2.11: Rescue of *pif1-2* chlorophyll biosynthetic phenotypes in the transgenic seedlings expressing wild type and truncated versions of PIF1. A) Chlorophyll content in wild type, *pif1-2* and the transgenic seedlings. Two independent LUC-PIF1 N-terminal (N150 line #9 and line #4) and C-terinal (C327 line #5 and line #15) truncated transgenic lines created in the *pif1-2* background were grown with the wild type, *pif1-2* mutant and LP line for chlorophyll measurements as described (Huq *et al*., 2004). Means ± SE of three biological replicates are shown. B) Transgenic, wild type and *pif1-2* mutant seedlings were grown for 6 days in the dark an then tansfered to white light for two days. Green seedlings were counted and expressed as percentage of green seedlings/genotype. Mean ± SE of three biological replicates are shown (n≥ 30). C) Visible phenotypes of the transgenic, wild type and *pif1-2* seedlings grown under 12h red (Rc, 15 $\mu molm^{-2}s^{-1}$) or far-red light (FRc, 12 $\mu molm^{-2}s^{-1}$)/ 12 h dark cycles for 4.5 days. White bar = 10 mm. D) Bargraph showing hypocotyls lengths of the transgenic, wild typea, and *pif1-2* seedlings grown as described in C. Means ± SE of thee biological repeats are shown (n≥30). E) Visible gravitropic phenotypes of the transgenic, wild type and *pif-2* sedlings grown in the dark. F) Percentage of hypocotyls in each genotype that displayed negative gravitropism in the dark. Means ±SE of three biological replicates are shown (n≥30). G) Visible phenotype of seed germination in transgenic, wild type and pif1-2 lines exposed to FR (3.2 $\mu molm^{-2}s^{-1}$) light for 5 min and then incubated in the dark for 6 days. Wild type seeds do not complete germination after FR light exposure.

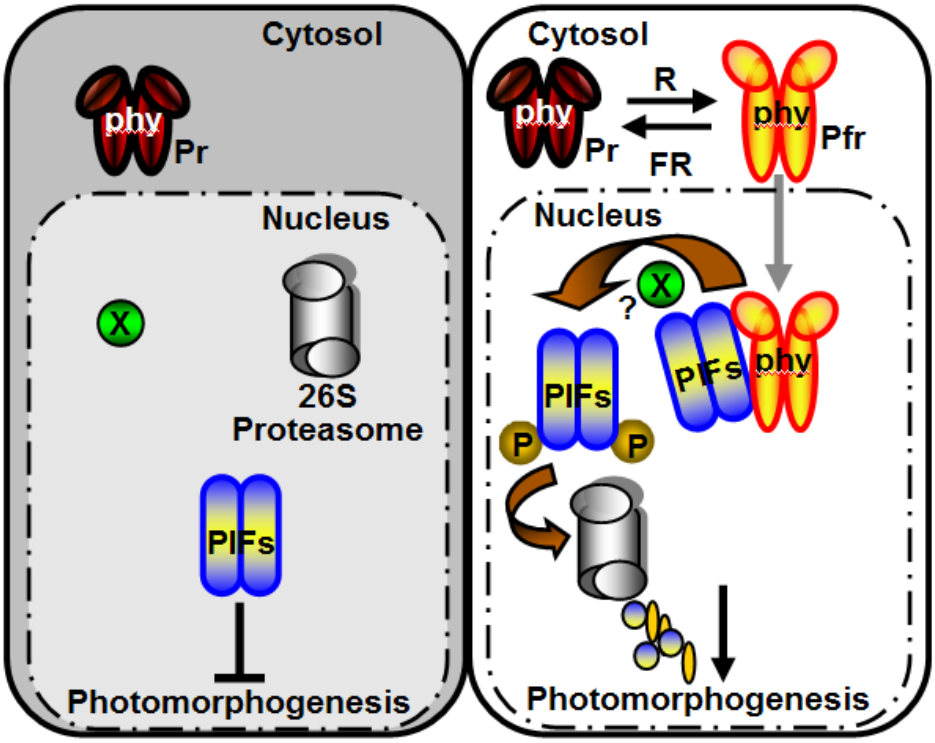


Figure 2.12: Simplified model of PIF function in phy signaling pathways. (Left) In the dark, phys are localized to the cytosol, while PIFs are constitutively localized to the nucleus and negatively regulate photomorphogenesis. (Right) Light signals promote nuclear migration of phys by inducing photo-conversion of the Pr form to the active Pfr form. In the nucleus, the photoactivated phys interact with PIFs, resulting in phosphorylation of PIF1 and other PIFs either directly or indirectly. The phosphorylated forms of PIFs are then poly-ubiquitinated by an ubiquitin ligase, and subsequently degraded by the 26S proteasome. The light-induced proteolytic removal of PIFs relieves the negative regulation, thus promoting photomorphogenesis. X, indicates an unknown factor that might be involved in the light-induced phosphorylation of PIFs. P, phosphorylated form. This figure is adapted and modified from Castillon *et al*., (2007).

CHAPTER III

Phytochromes Mediate the Phosphorylation and Degradation of PIF1 in Response to Blue Light [3]

SUMMARY

Photoactivated phytochromes interact with nuclear bHLH transcription factors called Phytochrome Interacting Factors (PIFs). PIFs have been shownt to negatively regulate photomorphogenesis both in the dark and light in Arabidopsis. The interaction with phytochromes induces the rapid phosphorylation and degradation of PIFs in response to both red and far-red light conditions to promote photomorphogenesis. Although phys have been shown to function under blue light conditions, the roles of PIFs under blue light have not been investigated in detail. Here we show that PIF1 negatively regulates photomorphogenesis at the seedling stage under blue light conditions. *pif1* seedlings displayed more open cotyledons and slightly reduced hypocotyl length compared to wild type under diurnal (12h light/12h dark) blue light conditions. Double mutant analyses demonstrated that *pif1phyA*, *pif1phyB*, *pif1cry1* and *pif1cry2* have enhanced cotyledon opening compared to the single photoreceptor mutants under diurnal blue light conditions. Blue light induced the rapid phosphorylation, poly-ubiquitination and degradation of PIF1 through the ubi/26S proteasomal pathway. PIF1 interacted with phyA and phyB in a blue light-dependent manner, and the interactions with phys are necessary for the blue light-induced degradation of PIF1. phyA played a dominant role under pulses of blue light, while phyA, phyB and phyD induced the degradation of PIF1

[3] Significant portions of this chapter appear in:

Castillón, A., Shen, H., and Huq, E. (2009). Blue light induces the degradation of the negative regulator Phytochrome Interacting Factor 1 to promote photomorphogenic development of Arabidopsis seedlings. *Genetics.* **182**, 161-171.

in an additive manner under prolonged continuous blue light conditions. Interestingly, the absence of cry1 and cry2 enhanced the degradation of PIF1 under blue light conditions. Taken together, these data suggest that PIF1 functions as a negative regulator of photomorphogenesis under blue light conditions, and that blue light-activated phys induce the degradation of PIF1 through the ubi/26S proteasomal pathway to promote photomorphogenesis.

INTRODUCTION

Light is a key environmental factor that regulates plant growth and development. Plants can track the intensity, color, direction, duration and overall day/night cycles of incoming light signals through an array of photoreceptors. These photoreceptors include: phytochromes (phys) that primarily respond to the red and far-red regions of the light spectrum; phototrophins (phot), cryptochromes (cry) and the ZTL/FKF1/LKP2 family of F-box proteins to monitor the UVA-blue light region; and an unidentified photoreceptor to respond to the UV-B light (Chen *et al.* 2004; Demarsy and Fankhauser 2008; Lin and Shalitin 2003; Schaefer and Nagy 2006). The coordinated function of these photoreceptors helps optimize growth and development throughout the plant's life cycle.

Five genes (*PHYA* to *PHYE*) have been found to encode phytochrome family members in *Arabidopsis thaliana* (Mathews and Sharrock 1997). phys exist in two photoreversible dimeric forms: a red light absorbing Pr form (biologically inactive), and a far-red light absorbing Pfr form (biologically active) (Schaefer and Nagy 2006). All phy family members are activated by red light, while phyA is activated by both red and far-red light signals (Quail 2007b). phy responses have been classified into three modes: very low fluence response (VLFR), low fluence response (LFR) and high irradiance response (HIR). VLFR responses achieve saturation by exposure to a brief pulse of light and are not photoreversible. LFR are red/far-red reversible responses induced by low light

intensities, and HIR responses are intensity dependent, non-photoreversible responses to high light intensities (Casal *et al.* 1998).

The phytochrome family members are differentially regulated at the posttranslational level and subcellular localization in response to light. For example, phyA is unstable under light and is the most abundant phytochrome in dark-grown seedlings, while phyB - phyE are relatively stable under light and are present in light-grown plants (Whitelam and Halliday 2007). Photoactivation of phys triggers a conformational change that induces the phys to be translocated into the nucleus (Fankhauser and Chen 2008). The light-triggered nuclear translocation has been shown to be necessary for the biological functions of both phyA and phyB (Huq *et al.* 2003; Matsushita *et al.* 2003; Rösler *et al.* 2007). However, cytosolic phyA has been shown to regulate negative gravitropism under blue light, as well as red light-induced enhancement of the blue light-mediated phototropism (Rösler *et al.* 2007). phys interact with a variety of nuclear proteins, and initiate a signal transduction pathway that ultimately regulates ~10% of the genome to promote photomorphogenesis (Jiao *et al.* 2007; Quail 2007a; Quail 2007b; Whitelam and Halliday 2007).

Once translocated into the nucleus of the cell, the phytochromes interact with a group of constitutively nuclear-localized basic helix-loop-helix transcription factors called Phytochrome Interacting Factors (PIFs) (Bae and Choi 2008; Castillon *et al.* 2007; Leivar *et al.* 2008a). PIFs interact with the biologically active Pfr forms of phyA and phyB using two discrete motifs, namely, the APB (active phyB binding motif) and APA (active phyA binding motif) that are present at the N-terminus of PIFs. PIFs have been shown to act as negative regulators of photomorphogenesis both in the dark and in light (Bae and Choi 2008; Castillon *et al.* 2007; Leivar *et al.* 2008a; Leivar *et al.* 2008b; Shen *et al.* 2008). To remove this negative regulation, the light-activated Pfr forms of phys physically interact with the PIFs, and induce the phosphorylation, polyubiquitination and

degradation of PIFs by the 26S proteasome-mediated pathway, and thereby promote photomorphogenesis. Strikingly, direct physical interactions with phys are necessary but not sufficient for the light-induced phosphorylation and degradation of PIFs (Al-Sady *et al.* 2006; Shen *et al.* 2008).

Although phytchromes are traditionally viewed as functioning under red and far-red light conditions, they have also been shown to function under blue light conditions (Casal 2000; Lin 2000). The absorption and action spectra for phys show a distinct peak in the blue light region (Mancinelli 1994; Rockwell *et al.* 2006; Shinomura *et al.* 1996; Vierstra and Quail 1983). Genetic evidence demonstrated that the phy and cry family members display both synergistic and antagonistic behavior at the seedling as well as adult stages. Analyses of photoreceptor mutants demonstrated that, under prolonged light exposure, both phyA and phyB regulate blue light-mediated seedling de-etiolation in an overlapping manner with cry1 and cry2 (Casal and Mazzella 1998; Neff and Chory 1998). phys and crys also displayed synergistic action in regulating blue light induced anthocyanin production and root greening at the seedling stage (Usami *et al.* 2007). However, phyB has been shown to oppose the cry1/phyA-mediated inhibition of hypocotyl elongation under blue light conditions (Folta and Spalding 2001). phyB and cry2 antagonistically regulate flowering time, while phyA and cry2 promote flowering time under long days (Lin 2000; Mockler *et al.* 1999). These photoreceptors also function to entrain the circadian clock (Somers *et al.* 1998), which independently control seedling de-etiolation and flowering time (Imaizumi and Kay 2006; McClung 2008; Nozue *et al.* 2007).

Members of the phy and cry families have been shown to physically interact with each other *in vivo*. For example, phyA interacts with cry1 (Ahmad *et al.* 1998), while phyB binds with cry2 (Mas *et al.* 2000). phyB, cry1 and cry2 have been shown to interact with a common signaling partner, CONSTITUTIVE PHOTOMORPHOGENIC 1 (COP1)

(Yang *et al.* 2001), suggesting that both photoreceptor families might directly inhibit the negative regulator COP1 to promote photomorphogenesis.

Although the physiological roles of phys have been investigated under blue light conditions, the roles of phy signaling factors in blue light are less understood. HFR1, a bHLH factor isolated as a positive regulator of FR-specific pathway, functions positively in a blue light signaling pathway (Duek and Fankhauser 2003). PIF4, a phyB-interacting bHLH factor, negatively regulates blue light signaling (Kang and Ni 2006). However, the molecular details of how PIF4 and/or other PIFs are regulated by blue light are still unknown. Here we show that PIF1, the PIF family member with the highest affinity for both phyA and phyB, functions negatively to repress seedling de-etiolation under blue light conditions. In addition, we show that blue light-activated phys induce the phosphorylation, polyubiquitination and subsequent degradation of PIF1 through the ubi/26S proteasomal pathway to promote photomorphogenesis.

MATERIALS AND METHODS

Plant growth conditions and phenotypic analyses

Plants were grown in Metro-Mix 200 soil (Sun Gro Horticulture, Bellevue, WA) under 24 hour light at 24°C ± 0.5°C. Monochromatic blue light treatments were performed in growth chambers equipped with light emitting diodes (LEDs) (Model E30LED, Percival Scientific, Madison, WI) as described (Shen *et al.* 2005). Light fluence rates were measured using a spectroradiometer (Model EPP2000, StellarNet Inc., Tampa, FL) as described (Shen *et al.* 2005). The wavelength specificity of the LED light sources is shown in Appendix 1. For transgenic plants, the 35S:LUC-PIF1 (LP), 35S:LUC-PIF1-3M (LP-3M) and 35S:TAP-PIF1 (TP) lines were generated as described (Moon *et al.* 2008; Shen *et al.* 2008; Shen *et al.* 2005). Seeds were surface sterilized and plated on

Murashige-Skoog (MS) growth medium (GM) containing 0.9% agar without sucrose (GM-Suc) as described (Shen *et al.* 2005). After 3-4 days of stratification at 4ºC in the dark, seeds were exposed to 3 hours white light at room temperature to induce germination before placing them in the dark or under continuous blue light or under diurnal (12h light/12h dark) blue light conditions for another 3 days.

Cotyledon angles were measured by gently placing the seedlings on adhesive tape facing upward. Digital photographs were taken through the dissection microscope and the angle formed between the two cotyledon tips was measured with the angle tool of ImageJ (1.37v, Wayne Rasband, NIH). Measurements for hypocotyl length were performed with ImageJ using the segmented line selections tool.

Protein extraction and Western blotting

Protein extraction and Western blotting were performed as described (Shen *et al.* 2008). Briefly, for blue light-mediated degradation, four day-old dark-grown seedlings were exposed to a pulse of 10 μmol/m^2 or 30 μmol/m^2 and kept in the dark for the indicated time periods. For the experiments requiring exposure to continuous blue light, dark-grown seedlings were exposed to 10 μmol$m^{-2}s^{-1}$ of blue light for the indicated time periods before harvesting for protein extraction. 0.2 g of tissue was collected and ground in 1 mL of extraction buffer: 0.1M Tris-HCl pH 6.8, 20% glycerol, 5% SDS, 0.01 M MG132, 0.2 M DTT, 2mM PMSF, and 1X proteinase inhibitors (Roche, complete mini, #11836170001) and boiled for 2 minutes. Samples were run on an 8% SDS-PAGE gel and blotted onto PVDF membrane. Another gel was run in parallel as loading control. The Western blot procedure was carried out according to manufacturer's instruction using KPL Protein Detector kit (#54-13-50) (KPL Inc., Gaithersburg, MA), utilizing 1:5,000 dilutions of anti-PIF1 antibody, and 1:2,500 anti-tubulin (Sigma-Aldrich Co., St. Louis, MO) as loading control. Peroxidase-labeled goat anti-rabbit antibody (KPL Inc., Gaithersburg, MA) in a 1:50,000 dilutions was used as secondary antibody. For the

immunoblot analyses to detect ubiquitination and phosphorylation, the membranes were blocked with 1 x TBST plus 2% non-fat milk buffer followed by incubation with different primary antibodies in 1 x TBST plus 0.5% non-fat milk buffer. Anti-Ubiquitin (1:700, Santa Cruz Biotechnology, Inc., Santa Cruz, CA) and Rabbit anti-c-MYC (1:800) (Sigma-Aldrich Co., St. Louis, MO) were used at 4°C overnight. For secondary antibody, peroxidase-labeled goat anti-rabbit antibody (1:4000, Pierce Biotechnology Inc., Rockford, IL) or anti-mouse IgG HRP conjugate (1:3300) (Promega, Madison, WI) was used, and membranes were developed with SuperSignal West Pico Chemiluminescent substrate kit (Pierce Biotechnology Inc., Rockford, IL).

Immunoprecipitation and alkaline phosphatase treatment

Immunoprecipitation and alkaline phosphatase treatment were performed essentially as described (Shen *et al.* 2008). Briefly, for pretreatment with MG132, 4 day-old dark-grown TAP-PIF1 seedlings were transferred into MS-suc liquid media containing 30 µM MG132 or equal volume of solvent control DMSO and incubated in the dark for 5 hours. Total proteins were extracted from ~0.4 g seedlings (either kept in darkness or treated with pulses of blue light followed by dark) with 1 mL denaturing buffer (100 mM NaH_2PO_4, 10 mM Tris [pH 8.0], 100 mM NaCl, 8 M urea, 0.05% Tween-20, 1x Protease inhibitor cocktail [F. Hoffmann-La Roche Ltd, Basel, Switzerland], 2 mM PMSF, 10 µM MG132, 25 mM β-GP, 10 mM NaF, 2 mM Na-orthovanadate and 100 nM calyculin A) and cleared by centrifugation at 14,000 rpm for 15 minutes at 4ºC. TAP-PIF1 was immunoprecipitated from supernatants with Ni-NTA magnetic agarose beads (Qiagen Inc., Valencia, CA) following incubation for 3 hours at 4ºC. After washing, the pellet was resuspended in 100 µl CIAP reaction buffer and then treated either with 100 U CIAP (F. Hoffmann-La Roche Ltd, Basel, Switzerland) or the same amount of boiled CIAP or without enzyme for 60 minutes at 37ºC. Pellets were washed with PBS buffer, heated at 65ºC in 1x SDS-Laemmli buffer for 5 minutes and subjected to Western blot analysis with anti-c-MYC or anti-ubiquitin antibody as described above.

Luciferase assay

Luciferase assays were performed as described (Shen *et al.* 2008; Shen *et al.* 2005). Briefly, samples were collected in liquid nitrogen and total protein was extracted using 1x Luciferase Cell Culture Lysis Reagent (CCLR) (Promega, Madison, WI) with 2mM PMSF and 1x complete protease inhibitor cocktail (F. Hoffmann-La Roche Ltd, Basel, Switzerland). For cycloheximide chase assays, 4 day-old dark-grown seedlings were pretreated with 50 µM cycloheximide in MS-Suc liquid medium for 3 hours in the dark as described (Shen *et al.* 2005). After pretreatment, the seedlings were exposed to a pulse of blue light (30 $\mu molm^{-2}$) and kept in darkness before harvesting for the time points indicated in the figure.

Light-dependent yeast-2-hybrid assays

Light-dependent yeast-two-hybrid assays were performed as described (Shimizu-Sato *et al.* 2002), except the yeast cells were exposed to pulses of blue light (30 or 3600 $\mu molm^{-2}$). Briefly, yeast cells (Y187) transformed with different constructs were grown overnight in synthetic dropout media with 25 µM PCB in the dark. After adding YPAD media, these cultures were either kept in the dark or exposed to a pulse of blue light and returned to darkness for additional three hours before assaying for LacZ reporter activity.

Isolation of RNA and RT-PCR

Total RNA was isolated using RNeasy Plant Mini Kit (Qiagen, Valencia, CA) from 4 day-old wild type Col-0 and *pif1-2* mutant seedlings treated for different time periods under blue light (25 $\mu molm^{-2}s^{-1}$). For RT-PCR, total RNA was treated with DNase I to remove genomic DNA. One µg of total RNA was reverse transcribed using the RT-PCR kit from Invitrogen (Invitrogen Life Science, Carlsbad, CA), and the first-strand cDNA was used as template for PCR amplification. For semi-quantitative gene expression, cDNAs were diluted to 40 µl with water and 1µl of diluted cDNA was used for PCR-

amplification of *PIF1* (forward 5'- CGAGATAACCGGTACATCGTCATC-3' and reverse 5'- CATCATTGGCATCATTCCAC-3'), *HY5* (forward 5'-GCTGCAAGCTCTTTACCATC-3' and reverse 5'-AGCATCTGGTTCTCGTTCTG-3'), *CHS* (forward 5'-TCGGTCAGGCTCTTTTCAGT-3' and reverse 5'-TGTCGCCCTCATCTTCTCTT-3') and *UBQ10* (forward 5'-GATCTTTGCCGGAAAACAATTGGAGGATGGT-3' and reverse 5'-CGACTTGTCATTAGAAAGAAAGAGATAACAGG-3') fragments using gene-specific primers. The *UBQ10* fragment was used as a control to normalize the amount of cDNA used. For all cDNAs, the exponential range of amplification cycles for each gene was determined experimentally. Then 26 (*PIF1*), 27 (*HY5*), 24 (*CHS*) and 27 (*UBQ10*) cycles were used for the RT-PCR experiments. Two biological repeats were carried out for each gene. PCR products were separated on agarose gel with ethidium bromide and imaged under UV light with Alpha Innotech Imager.

RESULTS

PIF1 negatively regulates seedling de-etiolation under diurnal blue light conditions

Under continuous blue light conditions, de-etiolation phenotypes of the *pif1* mutant seedlings were similar to that of the wild type (Figure 3.1). However, under diurnal blue light conditions, both alleles of *pif1* mutant seedlings displayed hypersensitive phenotype compared to wild type seedlings. Fluence rate response curves demonstrated that the angle between the cotyledons is significantly higher for *pif1* seedlings compared to wild type seedlings especially at lower fluence rates (Figure 3.2 A, C, D). Hypocotyl lengths for the *pif1* seedlings were also slightly shorter than the wild type seedlings (Figure 3.2 B, C). However, the cotyledon areas of *pif1* mutant and wild type seedlings were similar under these conditions (data not shown). These data suggest that PIF1 negatively regulates blue light signaling under diurnal conditions.

The absence of PIF1 in *phyA, phyB, cry1* and *cry2* mutant backgrounds enhances photomorphogenic responses of these single photoreceptor mutants under diurnal blue light conditions

In order to determine whether the hypersensitive phenotypes observed for *pif1* single mutants were phy or cry-dependent, double mutant combinations of *phyApif1, phyBpif1, cry1pif1* and *cry2pif1* were created by crossing the null allele of *pif1* (*pif1-2)* with different photoreceptor mutants. Seedling de-etiolation phenotypes including hypocotyl lengths and cotyledon angles were measured under a range of continuous and diurnal blue light conditions. Under diurnal blue light conditions, all four photoreceptor single mutants (*phyA, phyB, cry1* and *cry2*) displayed hyposensitive responses in suppression of hypocotyl elongation and expansion of cotyledon angles compared to the wild type seedlings under a range of blue light intensities (Figure 3.3 A-L). Strikingly, *pif1* mutant suppressed all the above phenotypes of the *phyA, phyB, cry1* and *cry2* single mutants in varying degrees under these conditions (Figure 3.3 A-L). The cotyledon angles of the *phyApif1* and *cry1pif1* double mutants were similar to that of the wild type seedlings under a wide range of blue light intensities (Figure 3.3 A-C, G-I). Under continuous blue light conditions, all four photoreceptor mutants displayed hyposensitive phenotypes in response to increasing light intensities (Figure 3.4). Under these conditions, *pif1* suppressed the cotyledon angle phenotypes of the *phyA* and *phyB* mutant completely, but did not suppress the cotyledon angle phenotypes of the *cry1* and *cry2* mutants. *pif1* suppressed the long hypocotyl phenotype of the *phyB* mutant, but did not suppress the long hypocotyl phenotypes of the *phyA, cry1* and *cry2* mutants under these conditions. These data suggest that PIF1 might function under multiple photoreceptors in suppressing the blue light-induced photomorphogenesis at the seedling stage.

Blue light-regulated gene expression is unaffected in *pif1* seedlings compared to wild type

It has been previously determined that blue light regulates a distinct set of genes including *HY5* and *CHS* in a light-dependent manner (Jiao *et al.* 2003; Ma *et al.* 2001). To investigate whether PIF1 plays a role in blue light-induced gene expression, we performed RT-PCR analysis on *HY5* and *CHS* (Jiao *et al.* 2003; Ma *et al.* 2001). The results show that the expression of these genes is similar in both *pif1* and wild type seedlings under blue light conditions. However, both *HY5* and *CHS* are expressed at a slightly higher level in dark-grown *pif1* seedlings compared to wild type seedlings (Figure 3.5). These data suggest that PIF1 is not involved in the blue light-induced expression of *HY5* and *CHS*. By contrast, PIF1 might reduce the expression of these genes in the dark to repress photomorphogenesis.

PIF1 is post-translationally regulated under blue light through the ubi/26S-proteasome pathway

Previous reports have shown that PIF1 functions as a negative regulator of both red and far-red light mediated seedling deetiolation processes (Huq *et al.* 2004; Oh *et al.* 2004; Shen *et al.* 2005). Red and far-red light induces degradation of PIF1 to remove this negative regulation (Oh *et al.* 2006; Shen *et al.* 2008; Shen *et al.* 2005). Since PIF1 also functions as a negative regulator under blue light conditions, we investigated whether PIF1 is degraded under blue light conditions. Western blots using an anti-PIF1 antibody demonstrated that native PIF1 is rapidly degraded in response to a pulse of blue light (Figure 3.6 A). Reduced PIF1 level might be due to a rapid reduction in transcription and/or instability of the *PIF1* mRNA under blue light conditions. To determine if *PIF1* mRNA level was reduced in blue light, we measured *PIF1* mRNA levels from total RNA isolated from seedlings exposed to blue light for different time periods using semi-quantitative RT-PCR assays. Results show that the expression of *PIF1* under blue light is similar to that in the dark up to 30 min. However, *PIF1* expression is induced after 1 hour

of blue light exposure, and this induction is decreased in *phyAcry1cry2* seedlings compared to wild type seedlings (Figure 3.6 B). These data suggest that blue light induces rapid post-translational degradation of PIF1 to promote photomorphogenesis at the seedling stage.

In order to determine whether blue light-induced degradation of PIF1 is mediated by the ubi/26S proteasomal pathway an experiment involving proteasome inhibitor MG132 was carried out. PIF1 protein levels were measured from extracts prepared from seedlings pretreated with and without MG132 in the presence and absence of blue light exposure. Results show that MG132 strongly inhibited the blue light-induced degradation of PIF1 (Figure 3.6 C), suggesting that PIF1 degradation under the blue light conditions is mediated through the ubi/26S proteasomal pahway.

Blue light induces the rapid phosphorylation and ubiquitination of PIF1

Because PIF1 is rapidly phosphorylated and poly-ubiquitinated prior to degradation under both R and FR light conditions (Shen *et al.* 2008), an experiment was carried out to determine whether PIF1 is also phosphorylated and ubiquitinated under blue light conditions. Seedlings expressing a 35S:TAP-PIF1 fusion protein were exposed to a pulse of blue light (3600 $\mu molm^{-2}$) followed by incubation in darkness for 1 hour. Protein extraction, immunoprecipitation and subsequent Western blotting show PIF1 migrated as a diffuse band with a higher mobility shift than PIF1 isolated from dark samples, suggesting that PIF1 is post-translationally modified under blue light (Figure 3.7 A). To test whether this modification was due to the addition of phosphate groups, TAP-PIF1 was immunoprecipitated from samples exposed to blue light and treated with Calf Intestinal Alkaline Phosphatase (CIAP). After CIAP treatment, the diffuse band is reduced to a sharp single band of lower molecular weight indicating the removal of the phosphates. Performing this experiment with boiled CIAP showed no effect on the

diffuse band. These results demonstrate that PIF1 is phosphorylated in response to blue light.

To investigate whether PIF1 is ubiquitinated in response to blue light signals, Western blots of immunoprecipiated TAP-PIF1 samples were probed using anti-ubi antibody. Figure 3.7 B shows that TAP-PIF1 is ubiquitinated under blue light conditions. Both anti-myc (specific to TAP-PIF1) and anti-ubi antibodies detected high molecular weight bands, which are enhanced in the presence of the proteasomal inhibitor MG132. These ubiquitinated forms are only present in the light-exposed samples, but not from the dark samples. These results along with Figure 3.6 C suggest that PIF1 is ubiquitinated and degraded under blue light conditions through the ubi/26S proteasomal pathway.

phyA is responsible for PIF1 degradation under pulses of blue light

Crys and phys are predominantly responsible for regulating seedling de-etiolation under blue light conditions. To investigate which photoreceptor induces PIF1 degradation under blue light, Western blot analyses of native PIF1 levels in monogenic and multiple photoreceptor mutant combinations were carried out. Results show that while cry1 and cry2 are not necessary for PIF1 degradation, phyA is responsible for the complete degradation of PIF1 under pulses of blue light (Figure 3.8 A). However, prolonged exposure to continuous blue light induced strong degradation of PIF1 in the *phyA* background, suggesting other photoreceptors are also involved in PIF1 degradation under blue light conditions (Figure 3.9). To investigate whether cry1 and cry2 participate in blue light-induced degradation of PIF1 under prolonged light conditions, we performed Western blots of proteins extracts from *phyA* and *phyAcry1cry2* triple mutant seedlings. Interestingly, results show that PIF1 level is reduced in the *phyAcry1cry2* compared to the *phyA* single mutant seedlings, suggesting that the absence of both cry1 and cry2 destabilizes PIF1 under these conditions (Figure 3.8 B). To estimate the relative contribution of cry1 and cry2 in PIF1 degradation, we performed Western blots of protein

extracts from *phyA*, *phyAcry1*, *phyAcry2* and *phyAcry1cry2* seedlings grown under continuous blue light. PIF1 is slightly less stable in *phyAcry1* and *phyAcry2* compared to *phyA* single mutant (Figure 3.8 C). However, PIF1 is completely degraded in the *phyAcry1cry2* triple mutant compared to either *phyAcry1* or *phyAcry2*, suggesting that the absence of both cry1 and cry2 synergistically destabilizes PIF1 under blue light.

PIF1 is degraded under blue light in a phy-dependent manner

Due to increased degradation of PIF1 in *phyAcry1cry2* seedlings compared to that in *phyA* seedlings under prolonged blue light conditions, we focused our attention on single and higher order *phy* mutant seedlings. A Western blot of protein extracts from *phyA*, *phyAB* and *phyABD* seedlings exposed to continuous blue light demonstrated that PIF1 is slightly more stable in the *phyAB* double mutant background compared to *phyA* single mutant background (Figure 3.8 D). In addition, PIF1 is completely stable in the *phyABD* triple mutant background under these conditions. These data suggest that all three photoreceptors (phyABD) are necessary for the blue light-induced degradation of PIF1 in an additive manner.

PIF1 interacts with phyA and phyB in a blue light-dependent manner

Because PIF1 is degraded under blue light in a phy-dependent manner, a yeast-two-hybrid experiment was set up to determine whether PIF1 can interact with phyA and phyB under blue light using the light-dependent yeast-two-hybrid assays as described (Shimizu-Sato *et al.* 2002). Results show that PIF1 can interact with the full-length phyA and the N-terminal half of phyB (phyB-NT) in a blue light-dependent manner (Figure 3.10 A). Exposure of 30 $\mu molm^{-2}$ of blue light induced interaction of PIF1 with phyA significantly higher than the dark controls. However, exposure of 3600 $\mu molm^{-2}$ of blue light induced strong interactions between PIF1 and either phyA or phyB-NT. These data suggest that PIF1 binds to both phyA and phyB under blue light conditions.

Direct interactions with phys are necessary for the blue light-induced degradation of PIF1

Previously, it had been demonstrated that three amino acids (G47, L95 and N144) in PIF1 are critical for interaction with the Pfr forms of phyA and phyB (Shen *et al.* 2008). Moreover, phy-interaction is necessary for PIF1 degradation under red light conditions, since a triple mutant form of PIF1 fusion protein (LUC-PIF1-3M), that has reduced affinity for both phyA and phyB (Figure 3.10 B), showed reduced degradation compared to wild type LUC-PIF1 fusion protein (Shen *et al.* 2008). Using these transgenic lines, we determined the blue light-induced degradation pattern of the triple mutant form of PIF1 and compared that to the wild type LUC-PIF1 degradation pattern using a cycloheximide chase assay as previously described (Shen *et al.* 2008). Results show that in blue light, the rate of degradation of LUC-PIF1 is much higher compared to the LUC-PIF1-3M degradation rate (Figure 3.10 B, C), suggesting that phy-interaction is necessary for the blue light-induced degradation of PIF1.

In order to determine whether phy-interaction is sufficient for the blue light-induced degradation of PIF1, we measured the level of two truncated LUC-PIF1 fusion proteins (1-150 amino acids necessary for PIF1 interaction with phys and 151-478 amino acids necessary for DNA binding and dimerization) in the dark and blue light conditions. Results showed that both isolated regions of PIF1 are stable under blue light conditions (Figure 3.10 D), suggesting that phy-interaction is not sufficient for the blue light-induced degradation of PIF1.

DISCUSSION

Previously PIFs have been characterized by their roles in red/far-red light signaling pathways, but they have not been characterized under blue light conditions. This study, provides genetic, biochemical and photobiological evidence that PIF1 is a negative regulator of blue-light mediated de-etiolation of Arabidopsis seedlings. Two alleles of monogenic *pif1* seedlings displayed significantly larger cotyledon angles and slightly shorter hypocotyls compared to wild type seedlings under a range of fluence rates of blue lights applied diurnally (Figure 3.2). Although the hypocotyl lengths of both *pif1* alleles were slightly shorter than the wild type seedlings in the dark as has been described previously (Huq *et al.* 2004; Shen *et al.* 2008), both *pif1* alleles did not display any cotyledon opening when grown in the dark for four days under these conditions. These data suggest that PIF1 functions as a negative regulator of the blue light signaling pathways.

The comparison among the *pif1* double mutants with either *phy* or *cry* single mutants revealed a more complex relationship. The absence of *pif1* in either a *phyA* or *phyB* or *cry1* or *cry2* single mutant background suppressed the respective photoreceptor mutant phenotypes either completely or partially under blue light conditions (Figures 3.3, 3.4). For example, the *phyA* single mutant displayed a strong hyposensitive phenotype under diurnal blue light conditions, while a *phyApif1* double mutant displayed an almost wild type phenotype under these conditions (Figure 3.3A). The relatively weak *pif1* phenotype in comparison to strong *phyApif1 or phyBpif1* or *cry1pif1* or *cry2pif1* double mutant phenotypes under blue light suggest that PIF1 might be a very subtle negative regulator of the blue light-mediated developmental processes. The negative role of PIF1 might be so subtle that its effect is very weak under normal strong photocurrents in the wild type

background. However, the negative effect of PIF1 is more penetrable when the photocurrent is reduced in any of the single photoreceptor mutant background.

Another plausible hypothesis is that PIF1 and all other PIFs might function negatively in the dark-grown seedlings as has been demonstrated recently (Leivar *et al.* 2008b; Shen *et al.* 2008). In this case, the negative role of PIF1 is very marginal or unpenetrable in the dark-grown monogenic *pif1* seedlings, but becomes more penetrant in the presence of light when the level of other PIFs is reduced due to their light-induced degradation. This hypothesis predicts that PIFs might be degraded in response to blue light signals, as previously observed under red/far-red light conditions (Castillon *et al.* 2007; Shen *et al.* 2008). To test this hypothesis, we determined PIF1 level in the dark-grown seedlings and dark-grown seedlings exposed to blue light conditions. Strikingly, PIF1 is rapidly degraded under these conditions through the ubi/26S proteasomal pathway (Figure 3.6). In addition, as observed under red and far-red light conditions, PIF1 is phosphorylated, poly-ubiquitinated and subsequently degraded under blue light conditions (Figure 3.7). Because PIF1 is degraded in response to a single pulse of blue light in a phyA-dependent manner (Figure 3.8 A), it is possible that this degradation is through the VLFR response of phyA, as previously observed under far-red light conditions (Shen *et al.* 2005). Taken together, these data are consistent with the proposal that PIF1 functions negatively in the dark to repress photomorphogenesis, and the blue light signals induce rapid degradation of PIF1 to remove this negative regulation, and thereby, promote photomorphogenesis.

It is striking that the *pif1* mutant displays hypersensitive phenotype under diurnal conditions (Figures 3.2, 3.3), but not under continuous blue light (Figures 3.1, 3.4). Previous results also demonstrated that *pif1* mutant is hypersensitive to red and far-red light applied diurnally, but not under continuous light (Oh *et al.* 2004; Shen *et al.* 2005). Although *PIF1* mRNA is not regulated by circadian clock or diurnal conditions (data not

shown), PIF1 protein level re-accumulates in the subsequent dark period after rapid degradation under red light and is also slightly diurnally regulated (Shen *et al.* 2005). It is possible that this diurnal regulation of PIF1 protein level might be one of the molecular bases for the differential phenotypes observed for *pif1* mutant under diurnal as opposed to continuous blue light conditions.

The data presented here also demonstrate that PIF1 interacts with phyA and phyB in a blue light-dependent manner (Figure 3.10 A). phyA plays a dominant role under pulses of blue light, while phyB and phyD regulate PIF1 level under prolonged blue light conditions in an additive manner (Figure 3.8 D). A reduced level of blue light-induced degradation of a mutant form of PIF1, that has lower affinity for both phyA and phyB, suggest that direct physical interactions with phys are necessary for PIF1 degradation under blue light conditions (Figure 3.10 B, C). Moreover, independent expression of two separate regions of PIF1 (1-150 amino acid region necessary for phy interaction, and 151-478 necessary for DNA binding and dimerization) as Luciferase fusion proteins in transgenic plants demonstrated that these isolated regions are not degraded under blue light conditions (Figure 3.10 D). Therefore, phy-interaction is necessary, but not sufficient for PIF1 degradation under blue light conditions. Combined, these data along with previous results suggest that PIF1 and other PIFs function as negative regulators of photomorphogenesis in the dark, and phys activated by all three monochromatic lights induce rapid degradation of PIFs to promote photomorphogenesis (Al-Sady *et al.* 2006; Lorrain *et al.* 2007; Shen *et al.* 2008; Shen *et al.* 2007).

Although crys are the primary photoreceptors for the blue light-induced seedling de-etiolation, they were not necessary for the blue light-induced degradation of PIF1. By contrast, the data show that the absence of both cry1 and cry2 destabilizes PIF1 under blue light conditions (Figure 3.8 A, B, C). Although other bHLH proteins have been shown to interact with cry1 and cry2 under blue light (Liu *et al.* 2008), PIF1 did not show

interaction with cry1 and cry2 in both yeast-two-hybrid assays and *in vivo* co-immunoprecipitation assay (data not shown). It is unclear how cry1 and cry2 stabilize PIF1 under blue light conditions. One possibility is that the physical interaction between crys and phys might titrate away phyA and phyB from direct interaction with PIF1. Alternatively, both phy and cry signaling pathways share the same downstream components that are necessary for PIF1 degradation. Therefore, in the absence of cry1 and cry2, higher level of either phys and/or phy signaling components induce increased degradation of PIF1 under blue light conditions. Moreover, the functional significance of PIF1 stabilization by crys is also unknown. Although phys and crys have been shown to function antagonistically in controlling flowering time, phenotypic analyses of monogenic and double mutant plants did not reveal any role of PIF1 in controlling flowering time (data not shown). Because there are multiple PIFs in Arabidopsis, it is possible that higher order *pif* mutants would be necessary to uncover the roles, if any, of PIFs in controlling flowering time.

In conclusion, although phys are best-known as red/far-red light sensing photoreceptors, our data and those of others establish broader and more direct roles of phys in regulating both morphological and molecular phenotypes under blue light signaling pathways. Therefore, phys might control photomorphorphogenesis under a broad spectrum of light conditions, while crys, phots and ZTL/FKF1/LKP2 family might regulate photomorphogenesis specifically under blue light conditions (Figure 3.11). Elucidation of the mechanisms by which these photoreceptors act synergistically and/or antagonistically to optimize photomorphogenic development will await further investigation.

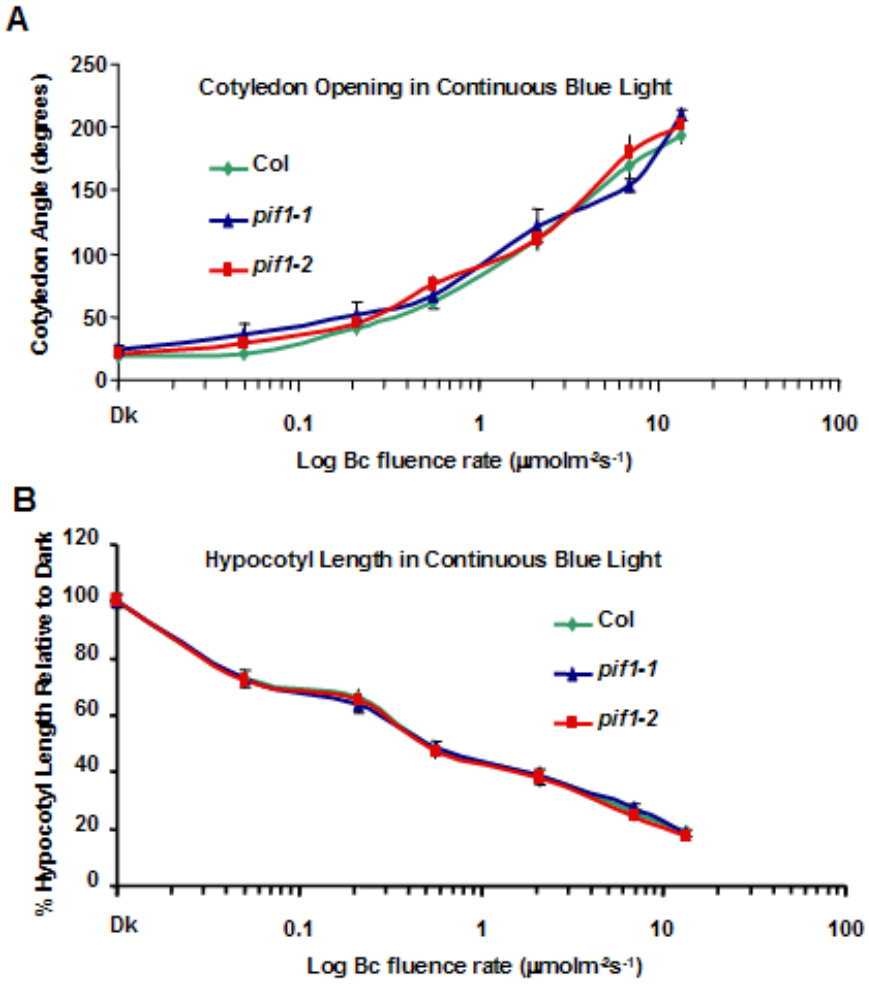


Figure 3.1: *pif1* seedlings displayed a wild type phenotype under continuous blue light conditions. Wild type, *pif1-1* and *pif1-2* seedlings were grown in dark or under increasing fluence rate of blue light. Cotyledon angles (A) and hypocotyl lengths (B) were measured using four day-old seedlings.

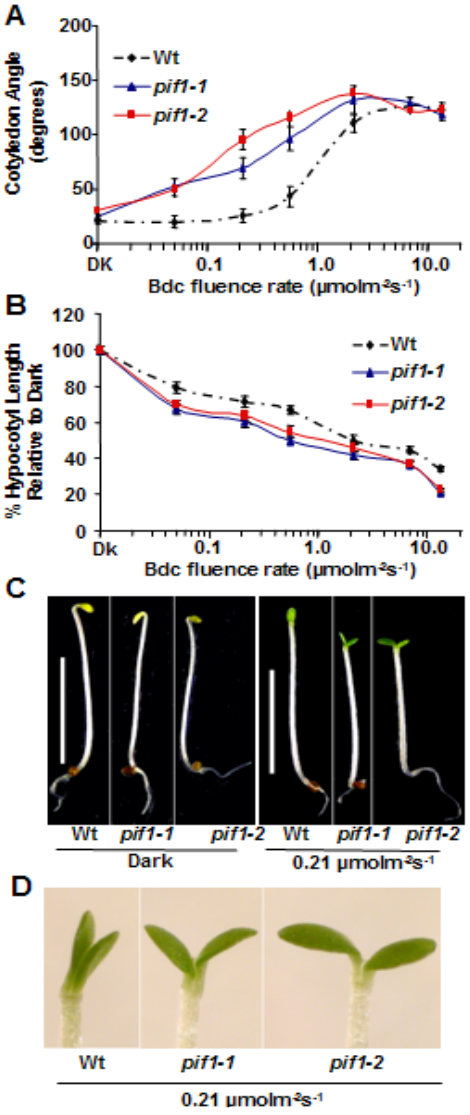


Figure 3.2: *pif1* seedlings are hypersensitive to blue light-induced seedling de-etiolation. Fluence-rate response curves of mean cotyledon angles (A) and hypocotyl lengths (B) of wild type (Col-0) and *pif1* alleles grown for four days under either dark or diurnal (12h light/12h dark) blue light conditions. Data are presented as mean $\pm$ SEM ($n \geq 30$, three replicates). Wt = wild type Col-0. (C) Photographs of seedlings grown under diurnal (12h light/12h dark) blue light conditions (0.21 µmolm^{-2}s^{-1}) and dark for four days. White bar = 5 mm. D) Enlarged photographs of the apical regions of wild type, *pif1-1* and *pif1-2* seedlings grown under conditions described in (C).

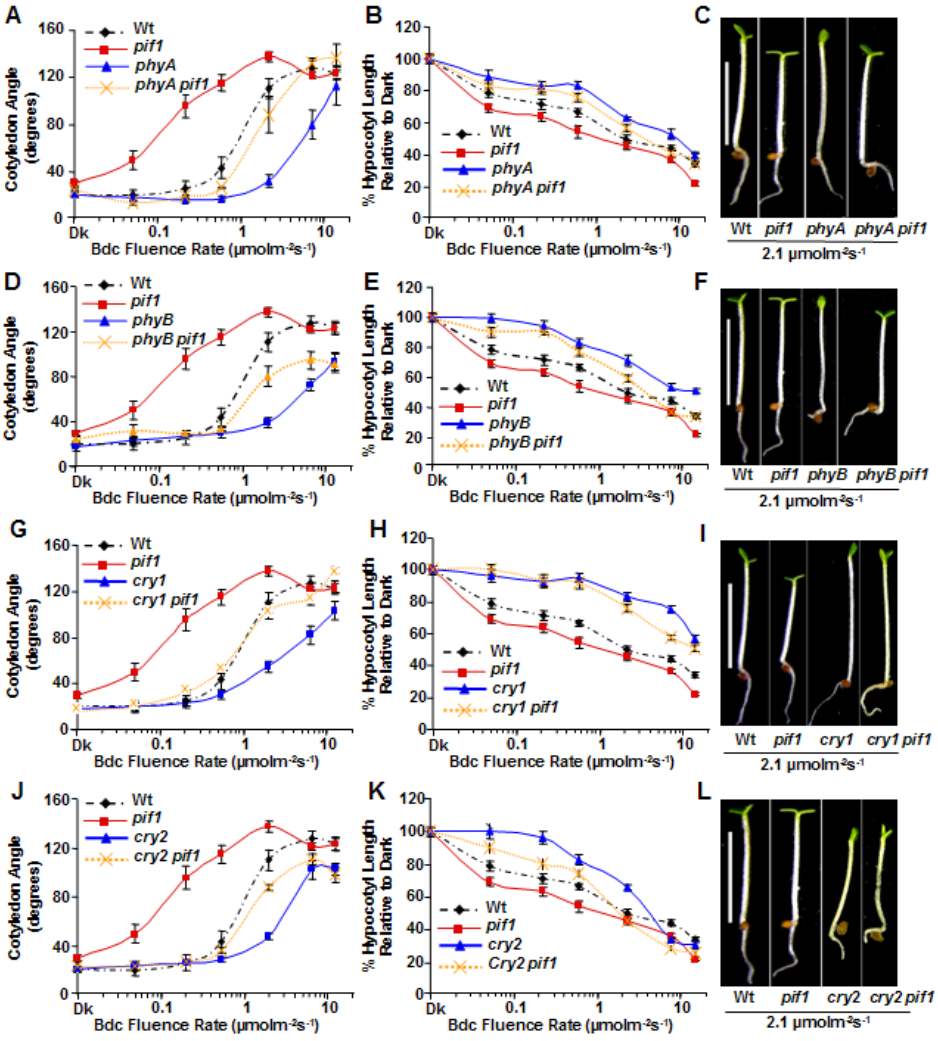


Figure 3.3: The absence of PIF1 in *phyA*, *phyB*, *cry1* and *cry2* mutant background enhances photomorphogenic responses under diurnal (12h light/12h dark) blue light conditions. Fluence-rate response curves of mean cotyledon angles (A,D,G,J) and hypocotyl lengths (B,E,H,K) of wt (Col-O), *pif1*, *pif1phyA* (ABC), *pif1phyB* (DEF), *pif1cry1* (GHI) and *pif1cry2* (JKL) grown for four days under either dark or diurnal (12h light/12h dark) blue light conditions. Hypocotyl lengths were normalized by setting the dark values to 100. Data are presented as mean $\pm$ SEM ($n \geq 30$, three replicates). (C,F,I,L) Photographs of seedlings of different genotypes grown under diurnal (12h light/12h dark) blue light conditions (1.2 µmolm^{-2}s^{-1}) for four days are shown. White bar = 5 mm.

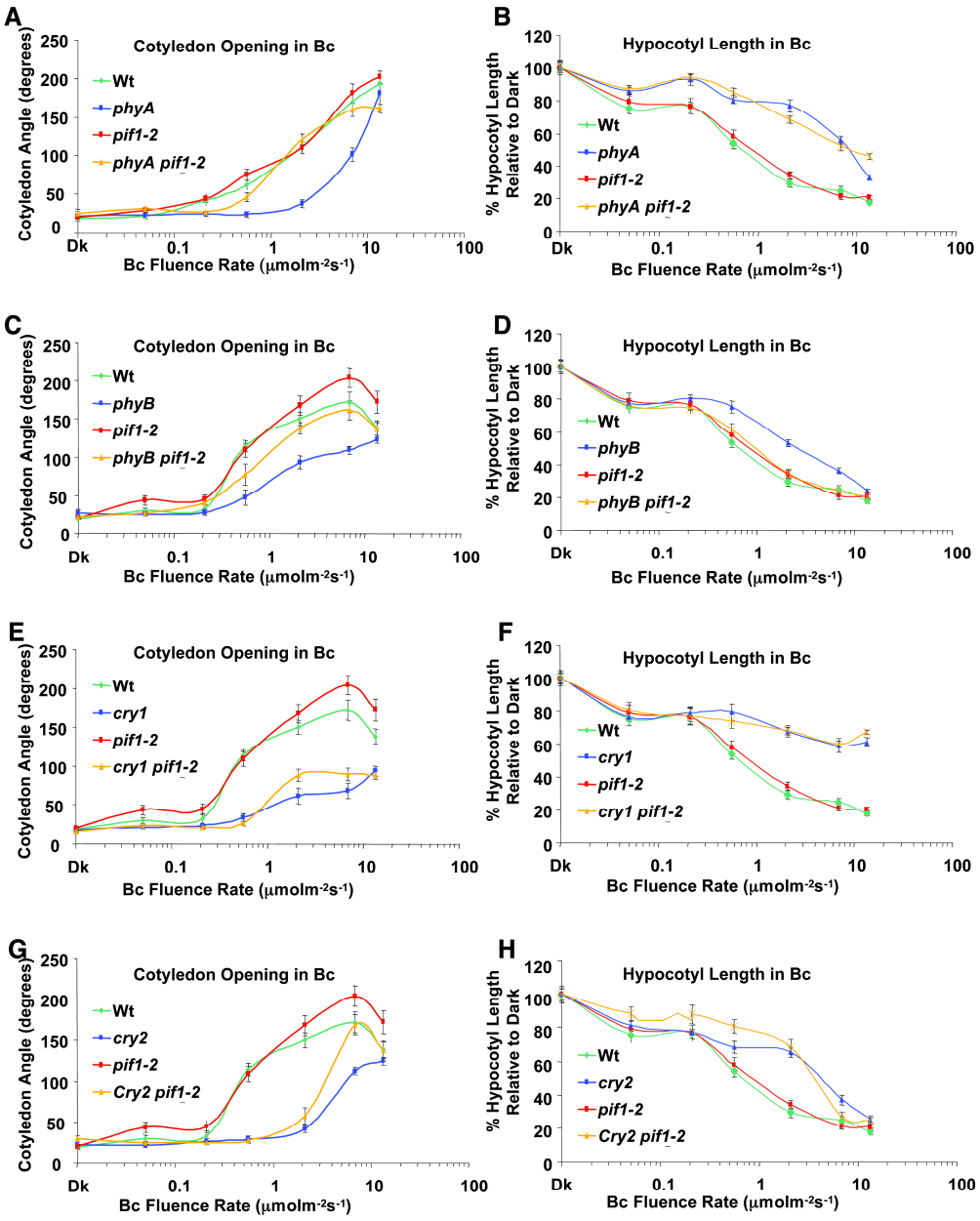


Figure 3.4: *pif1* suppresses the hyposensitive phenotypes of the *phyA*, *phyB*, *cry1* and *cry2* single photoreceptor mutants under continuous blue light conditions. Seedlings were grown under increasing fluence rates of blue light for four days, and cotyledon angles (A,C,E,G) and hypocotyl lengths (B,D,F,H) were measured.

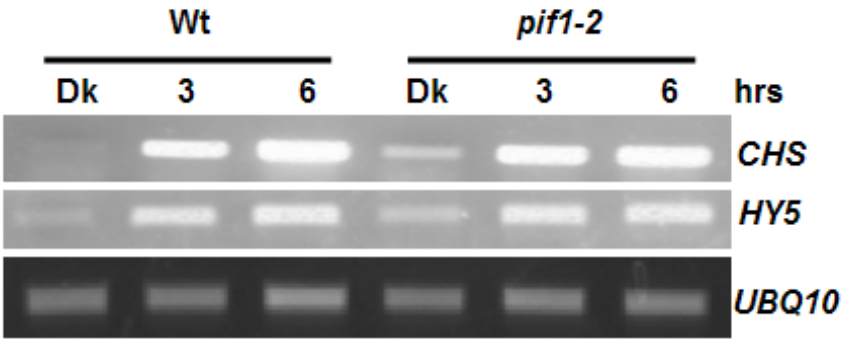


Figure 3.5: Blue light-regulated gene expression is unaffected in *pif1* mutant seedlings. Semi-quantitative RT-PCR assays for *HY5* and *CHS* using total RNA isolated from wild type and *pif1* mutants grown in the dark and dark-grown seedlings exposed to blue light (25 $\mu molm^{-2}s^{-1}$) for 3 and 6 hours.

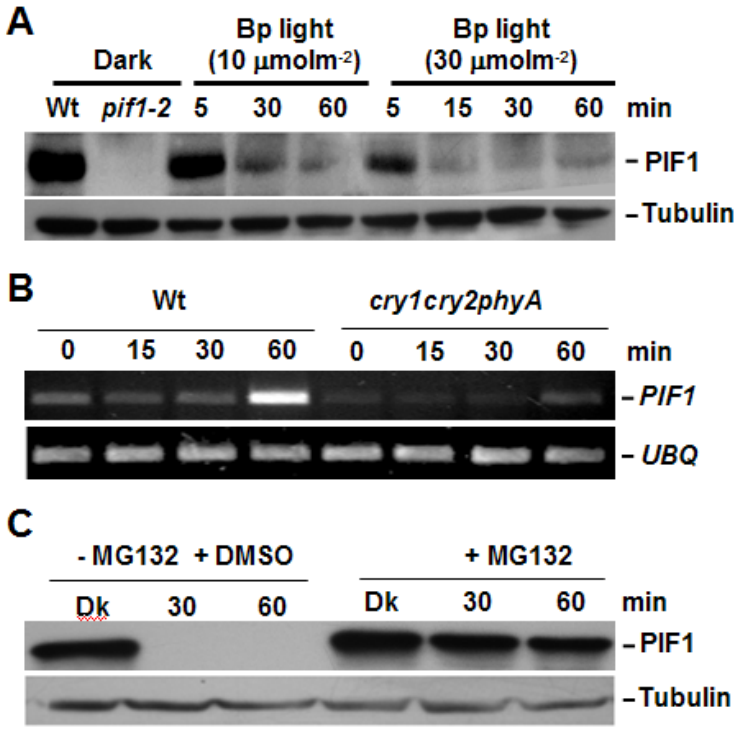


Figure 3.6: Blue light induces rapid degradation of PIF1 through the ubi/26S proteasomal pathway. A) Native PIF1 is rapidly degraded after exposure to a pulse of blue (Bp) light conditions. Four day-old dark-grown seedlings were exposed to Bp light (10 or 30 µmolm^{-2}) and then incubated in the dark for the time indicated before harvesting for protein extraction. As controls, protein extracts from dark-grown wild type and *pif1* seedlings are included in the first two lanes, respectively. Approximately 30 µg of total protein in each lane was separated on an 8% polyacrylamide gel, transferred to PVDF membrane and probed with anti-PIF1 antibody. A similar blot was probed with anti-tubulin antibody. The bands corresponding to PIF1 and tubulin are labeled. B) *PIF1* is slightly induced under blue light conditions. RT-PCR analyses of *PIF1* mRNA levels extracted from four-day old dark-grown seedlings or four-day old dark-grown seedlings exposed to continuous blue light (25 µmolm^{-2}s^{-1}) for the durations indicated. *UBQ10* was used a control for the RT-PCR assays. C) Blue light-induced degradation of PIF1 is mediated through the ubi/26S proteasomal pathway. Four day-old dark-grown seedlings were pretreated with or without MG132 (30 µM) for 5 hours before being exposed to Bp light (30 µmolm^{-2}) and then incubated in the dark for the durations indicated.

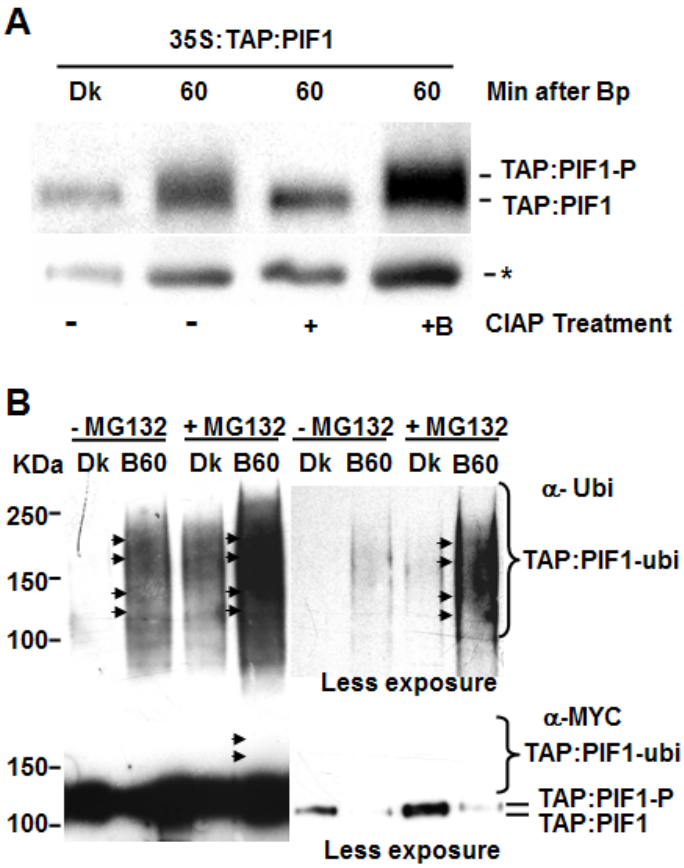


Figure 3.7: Blue light induces rapid phosphorylation and ubiquitination prior to degradation of PIF1. A) Blue light induces phosphorylation of PIF1. TAP-PIF1 was immunoprecipitated from protein extracts prepared using four day-old dark-grown 35S:TAP-PIF1 seedlings kept in the dark or exposed to Bp (30 $\mu molm^{-2}s^{-1}$ x 2 min = 3600 $\mu molm^{-2}$) followed by dark incubation. The immunoprecipitated pellets from the Bp-exposed samples were dissolved in buffer and incubated without (-) or with (+) native Calf Intestine Alkaline Phosphatase (CIAP) or with boiled CIAP (+B). Samples were then separated on 6.5% SDS-PAGE gels and Western blots probed with anti- MYC antibody. Asterisks denote cross-reacting bands. B) Blue light induces ubiquitination of PIF1. TAP-PIF1 was immunoprecipitated from protein extracts prepared using four day-old dark-grown seedlings either kept in the dark (Dk) or exposed briefly to Bp light (30 $\mu molm^{-2}s^{-1}$ x 2 min = 3600 $\mu molm^{-2}$). The immunoprecipitated samples were then separated on 6.5% SDS-PAGE gels and probed with anti-ubiquitin (Ubi) or anti-MYC antibodies. Arrows indicate ubiquitinated forms of PIF1. Data obtained by Hui Shen.

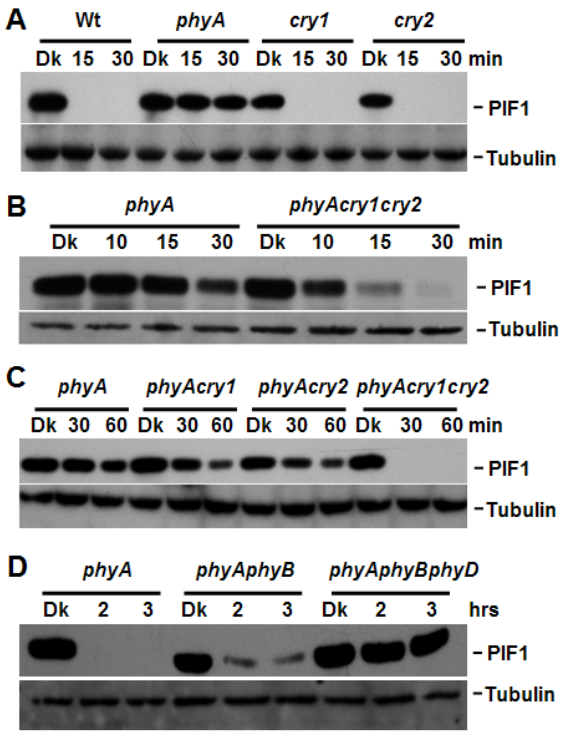


Figure 3.8: phyA is necessary for PIF1 degradation while cry1 and cry2 stabilizes PIF1 under blue light conditions. A) phyA mediates PIF1 degradation after exposure to a pulse of blue light. Four day-old dark-grown seedlings were exposed to a pulse of blue light (Bp, 10 μmolm^{-2}), and then incubated in the dark for the durations indicated before being harvested for protein extraction. B) PIF1 is less stable in *phyAcry1cry2* seedlings compared to *phyA* seedlings under continuous blue light conditions. Four day-old dark-grown seedlings were exposed to continuous blue light (Bc, 10 μmolm^{-2}s^{-1}), and then incubated in the dark for the durations indicated. C) Absence of cry1 and cry2 synergistically destabilizes PIF1 under continuous blue light conditions. Four day-old dark-grown seedlings were exposed to continuous blue light (Bc, 10 μmolm^{-2}s^{-1}), and then incubated in the dark for the durations indicated before being harvested for protein extraction. D) phyA plays a dominant role during the initial light exposure while phyB and phyD regulate PIF1 stability under prolonged light exposure. Western blots showing native PIF1 levels in *phyA*, *phyAB* and *phyABD* mutant backgrounds. Four day-old dark-grown seedlings were exposed to continuous blue light (Bc, 10 μmolm^{-2}s^{-1}) for the time indicated before harvesting for protein extraction. All three genotypes are in Ler ecotype.

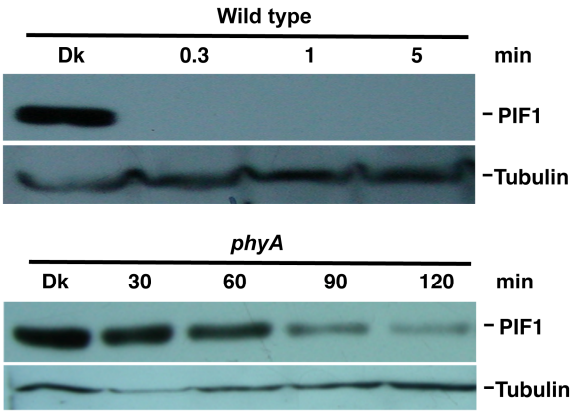


Figure 3.9: PIF1 is degraded in both wild type (top) and *phyA* (bottom) background under continuous blue light exposure. Four day-old dark-grown seedlings were exposed to continuous blue light (10 μmolm^{-2}s^{-1}) for the time indicated before harvesting for protein extraction and Western blot analysis.

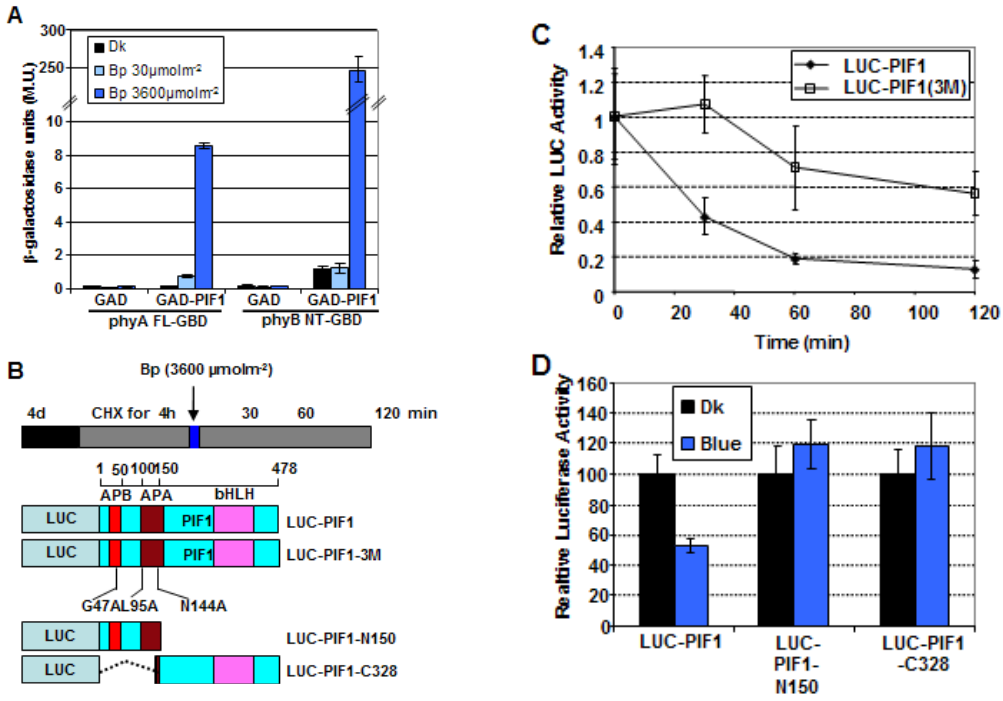


Figure 3.10: Interactions with the Pfr form of phyA and phyB are necessary for the light-induced degradation of PIF1. A) PIF1 interacts with phyA and phyB-NT in a blue light-dependent manner in quantitative yeast two-hybrid assays. LacZ assays were performed in triplicate and the data represent mean ± SE. Yeast cells were exposed to pulses of blue light as indicated, and then incubated in the dark for additional 3 hours before performing LacZ assay. M.U., Miller units. phyB-NT is the N-terminal half (1-621 amino acid) of phyB. B) (Top) Design of the cycloheximide chase assays. (Bottom) Schematic representation of the full-length, truncated and missense mutant forms of LUC-PIF1 fusion proteins used in the experiment. C) Relative Luciferase activity for phy-interaction deficient mutants was measured in 4-day-old dark-grown seedlings pretreated with cycloheximide (CHX) in the dark for 3 hour, exposed to a pulse of blue light (30 □molm^{-2}), and then incubated in the dark for the indicated time (min). Assays show the kinetics of degradation of LUC-PIF1-3M compared to wild type LUC-PIF1. LUC-PIF1-3M is deficient in both phyA and phyB interaction. Means ± SE of three biological replicates are shown. D) Relative Luciferase activity for the truncated versions of PIF1 fusion proteins compared to the wild type LUC-PIF1 fusion protein. Four day-old dark-grown seedlings were exposed to a pulse of blue light (30 □molm^{-2}) and then incubated in the dark for 60 minutes before harvesting for protein extraction and Luciferase assays.

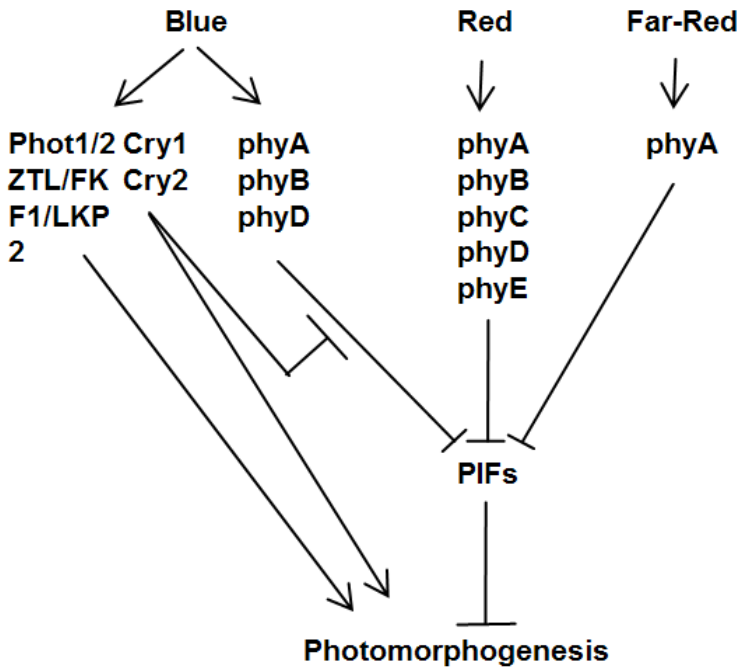


Figure 3.11: Simplified model of Arabidopsis photoreceptors' function in the light regulation of photomorphogenesis. cry1, cry2, phot1, phot2 and ZTL/FKF1/LKP2 family of photoreceptors perceive and respond to the blue region of the light spectrum, while phys perceive and respond to all three (blue, red and far-red) light signals. All five phys perceive and respond to red light signals, while phyA, phyB, phyD and possibly phyC/phyE respond to blue light signals. phyA is the sole photoreceptor for perceiving and responding to the far-red light signals. phys and crys may function synergistically and/or antagonistically to optimize photomorphogenesis under blue light signals.

CHAPTER IV

Combinatorial Control of Photomorphogenesis by the bHLH Class of Transcription Factors in Arabidopsis

SUMMARY

bHLH transcription factors function as homodimers and heterodimers. To identify PIF1-interacting proteins, we performed yeast two-hybrid screening using PIF1 as a bait and identified as interactors PIF1 itself, PIF3 and HFR1. In addition, PIF4 was identified as PIF1 interactor in quantitative yeast two-hybrid assays. PIF3 functions negatively and HFR1 functions positively in light signaling pathways in Arabidopsis. To understand the functional significance of their interaction with PIF1, *pif1pif3, pif1hfr1* and *pif3hfr1* double mutants were obtained and characterized phenotypically. The *pif1pif3* double mutant exhibited enhanced cotyledon opening and enhanced reduction in hypocotyl length under discontinuous blue light, when compared to the single mutants. This indicates an additive regulation of photomorphogenesis by PIF1 and PIF3 under these conditions. *pif1hfr1* double mutants exhibited the phenotypes of either the *pif1* single mutant or the *hfr1* single mutant depending on the light conditions assayed. The *pif1hfr1* double mutant presented the characteristic phenotype of *pif1,* considering seed germination after exposure to far-red light. The *pif1hfr1* mutant showed the hyposensitive phenotype characteristic of *hfr1,* under continuous far-red light and blue light. The *pif1hfr1* double mutant also exhibited exaggerated shade avoidance responses similar to the *hfr1* mutant. These data suggest a combinatorial control of photomorphogenesis by the positively and negatively acting bHLH proteins in response to light in Arabidopsis.

INTRODUCTION

Plants use light signals to gather information about their surrounding environment and modulate their growth and development accordingly. Light signals are perceived by at least four families of photoreceptors in plants: phytochromes (phys), cryptochromes (crys), phototropins (phots) and ZTL/FKF1/LKP2 family of F-box proteins. phys respond to a broad range of blue, red and far-red region of the light spectrum, while crys, phots and ZTL/FKF1/LKP2 monitor the UVA-blue region of the light spectrum. phys are encoded by a small group of genes in *Arabidopsis thaliana* (*phyA-phyE*) (Mathews and Sharrock, 1997). The phy holoproteins exist in two photoreversible forms: a red light absorbing Pr form (biologically inactive), and a far-red light absorbing Pfr form (biologically active) (Schaefer and Nagy, 2006). Light triggers a conformational change in phy holoproteins that induces the phy family members to translocate into the nucleus with differential kinetics (Kircher et al., 2002; Sakamoto and Nagatani, 1996). In the nucleus phys interact with a group of transcription factors called Phytochrome Interacting Factors (PIFs). PIFs have been shown to function as negative regulators of photomorphogenesis both in the dark and light. Light signals induce degradation of PIFs in a phy-dependent manner to remove this negative regulation, and thereby promote photomorphogenesis (Shen et al. 2008, Al Sady et al. 2006).

Although PIFs are highly homologous proteins, monogenic *pif* mutants displayed distinct morphological phenotypes. For example, *pif1* and *pif3-pif7* single mutants displayed short hypocotyl phenotypes under red and/or far-red light conditions. In addition, PIF1 acts as a repressor of light-induced seed germination and chlorophyll accumulation. *pif1* mutants germinate after far-red light exposure due to a mis-regulation of various hormone biosynthetic and signaling genes (Oh et al., 2009). *pif1* seedlings exhibit photooxidative damage (bleaching) and fail to green when dark-grown seedlings

are transferred to light primarily due to mis-regulation of chlorophyll biosynthetic genes in the dark (Huq et al., 2004, Moon et al, 2008). A quadruple *pif1pif3pif4pif5* mutant displayed constitutively photomorphogenic phenotypes in the dark, suggesting that PIFs promote skotomorphogenic growth pattern in the dark.

PIFs belong to the basic helix-loop-helix (bHLH) superfamily of transcription factors (Heim et al., 2003; Toledo-Ortiz et al., 2003). bHLH factors are characterized by two distinct regions: the helix-loop-helix region (HLH), consisting in approximately 60 amino acids and a basic region of approximately 15 amino acids involved in binding to the target DNA. bHLH transcription factors bind DNA as dimers, either homodimers and/or heterodimers. The partners that form the dimer interact with each other through the HLH domain. The dimer of bHLH factors can bind to cis-acting regulatory elements found in the promoter regions of target genes. Each member of the dimer contacts the DNA region through its basic region. The most common of these cis-elements is the E-box (5'-CANNTG-3'). E-boxes are classified into different types depending on the central two nucleotides, for example a G-box (5'-CACGTG-3'). It has been proposed that the nucleotide regions flanking the E/G-box also play a role in specifying which transcription factors bind to that region (Littlewood and Evans, 1988). In the case of bHLH factors that form heterodimers with multiple partners, each different combination would bind to slightly diverse promoter regions giving further specificity to the response.

PIF family members have been shown to form homodimers and/or heterodimers. For example, PIF3 can homodimerize with itself as well as heterodimerize with PIF4 (Toledo-Ortiz et al., 2003), and a non-PIF bHLH factor HFR1 (long hypocotyl in far-red). HFR1 is an atypical bHLH factor, functioning positively in far-red and blue light signaling pathways (Duek and Frankhauser, 2003; Fairchild et al., 2000). The PIF3-PIF3 homodimer as well as the PIF3-PIF4 heterodimer can bind to a G-box DNA sequence element in vitro (Toledo-Ortiz et al., 2003; Huq and Quail, 2002; Martinez-Garcia et al.,

2000). It is possible that the heterodimerization between different PIF family members increases the diversity of regulation of photomorphogenesis by PIFs.

Although PIF1 functions as a critical regulator of photomorphogenesis, homo- and hetero-dimerization of PIF1 with other PIFs and/or other bHLH proteins have not yet been shown. In this study, we show that PIF1 can interact with a group of bHLH proteins functioning both positively and negatively in regulating photomorphogenesis. The phenotypical analyses of *pif1pif3, pif1hfr1* and *pif3hfr1* double mutants under diverse light conditions are presented.

MATERIALS AND METHODS

Plant growth conditions, light treatments and phenotypic analyses

Plants were grown in Metro-Mix 200 soil (Sun Gro Horticulture, Bellevue, WA) under continuous light at 24°C ± 0.5°C. Seeds were sterilized with 10% bleach + 0.1% SDS for ten minutes, washed five times with water and then plated on Murashige-Skoog (MS) growth medium (GM) containing 0.9% agar without sucrose (GM-Suc). After 4 days of stratification at 4º C in the dark, seeds were exposed to 1.5 hours white light at room temperature to induce germination and kept in darkness for 23 hours. After this time period, the plates were transferred to growth chambers to receive red, far-red, or blue light treatments for additional 3 days. Monochromatic blue light treatments were performed in growth chambers equipped with light emitting diodes (LEDs) (Model E30LED, Percival Scientific, Madison, WI) as described (Shen *et al.* 2005). Light fluence rates were measured using a spectroradiometer (Model EPP2000, StellarNet Inc., Tampa, FL) as described (Shen *et al.* 2005).

For quantification of hypocotyl length, digital photographs of seedlings were taken and at least 30 seedlings were measured using the publicly available software ImageJ (1.37v, Wayne Rasband, NIH, http://rsb.info.nih.gov.ezproxy.lib.utexas.edu/ij/). Experiments were repeated at least three times. For measurements of cotyledon angles, seedlings were gently placed in an adhesive tape facing upwards. Cotyledon angles were measured as the angle formed between the two cotyledons tips with the angle tool of ImageJ. In the case of seedlings with cotyledon angles greater than 180^{o}, such as *cop1* mutants, the interior acute angle between the two cotyledons was measured and subtracted from 360^{o}.

Yeast two-hybrid Screening and Quantitative β-Galactosidase Assay

Procedures for the yeast two-hybrid screen and quantitative interaction assays were performed according to the manufacturers instructions (Matchmaker Two-Hybrid System, Clonetech Laboratories Inc., CA). Full length PIF1 as well as the following PIF1 fragments were cloned into pGBT9 to be assayed as baits: N50 (1-50 aa), N150 (1-150 aa), N280 (1-280 aa), C198 (280-478 aa), C328 (150-478 aa), and C428 (50-478 aa). The C328 fragment was selected as suitable bait for large scale screening, as this fragment did not show any transcriptional activation activity. A λACT cDNA library was converted to a pACT library according to the procedure described (Durfee et al., 1993). For the quantitative interaction assays, a truncated version of PIF3 (lacking the N-terminal 120 amino acids) as well as full length HFR1 were cloned into pGBT9 to be used as baits. Prey constructs of full length PIF1, HFR1 and PIF4 were constructed in pGAD424. Full length PIF3 was cloned into pGAD424 to be used as prey.

Germination Assay

The phyA mediated germination assay was performed as described (Oh et al., 2004) with modifications. 60 seeds of each phenotype surface sterilized. The seeds were

imbibed for one hour at 24^{o} C (including the sterilization time) and plated in MS-Sucrose. Immediately after plating, seeds were given a treatment of a total of 16 micromoles of far-red light to reverse the active phytochromes. Plates are wrapped in aluminum foil and kept in the darkness at 21˚C for 56 hours. After this period of time plates are exposed to different amounts of far-red light to induce germination. A plate with no light treatment is kept as a control. Plates are wrapped again and kept in the darkness at 21˚C for five days, after which germination is visually scored.

For the phyB mediated germination assay seeds are sterilized and plated as described for the phyA mediated assay and also treated with 16 micromoles of far-red light. Subsequently they are treated with different amounts of red light to induce germination. A plate with no light treatment is kept as a control. Plates are wrapped again and kept in the darkness at 21˚C for five days, after which germination is visually scored.

Co-Immunopreciptiation and DNA Binding Assay

PIF1 and PIF3 were cotranslated using the TnT system and co-immunoprecipitation assays were carried out as previously described (Toledo-Ortiz *et al.*, 2003; Huq *et al.*, 2002).

Shade Avoidance Response

The assay for the shade avoidance response was performed as described (Sessa et al., 2005) with some modifications. Seeds were surface sterilized and plated in MS media without sucrose. After 4 days of stratification at 4º C in the dark, seeds were exposed to 1.5 hours white light at room temperature to induce germination and kept in darkness for 23 hours. Seedlings were placed under short day light/dark cycles for 3 days then were transferred to light conditions of either low R to FR light ratio **(**red light 8 μmol/m^{2}s, far-red light 60 μmol/m^{2}s; and blue light 10 μmol/m^{2}s) or high R to FR **(**for red light 60

μmol/m^2s**;** for far-red 13 μmol/m^2s, and blue light 10 μmol/m^2s**)** light ratio for 5 days. For measurement of hypocotyl and petiole length, as well as leaf area, digital photographs of seedlings were taken and at least 30 seedlings were measured using the publicly available software ImageJ (1.37v, Wayne Rasband, NIH, http://rsb.info.nih.gov.ezproxy.lib.utexas.edu/ij/).

RESULTS

Screening for PIF1 interacting proteins

To identify proteins that interact with PIF1 and are possibly involved in the phytochrome signal transduction pathway, we carried out a yeast two-hybrid screen of an *Arabidopsis thaliana* cDNA library using PIF1 as bait. Full-length PIF1, being a transcription factor, activates the expression of *LacZ* and *HIS3* reporter genes by itself and thus it is not a suitable bait for the screen. Six PIF1 deletion constructs were fused to GAL4 DNA binding domain and assayed for transcriptional activation in yeast to find a suitable bait (Figure 4.1 A). These constructs were transformed into AH109 yeast strain and assayed for growth in YPD medium lacking histidine (Figure 4.1 B). All of the constructs, except C198 and C328, supported growth on media lacking histidine, and thus activate the expression of the *HIS3* gene without a prey. Fragment C328 was selected for screening as it was the largest fragment without transcriptional activation activity. After screening ~$2x10^6$ colonies on selective media lacking histidine and adenine, 29 positive colonies were selected. Two of the clones identified multiple times encode bHLH factors involved in photomorphogenesis, PIF3 (2 times) and HFR1 (7 times) as shown in Figure 4.2 A and B.

Quantitative β-galactosidase assays reveal a network of interactions among PIF1, PIF3, PIF4 and HFR1

Although PIF4 was not identified from the screen, it was included in the quantitative interaction assays based on its roles in photomorphogenesis. Growth of yeast colonies on selective media (Figure 4.3 A) and quantitative yeast two-hybrid assays (Figure 4.3 B) were performed among all the possible combinations among PIF1, PIF3, PIF4 and HFR1. Strong interactions were detected among PIF1 and the two other PIF family members, PIF3 and PIF4. Interactions among HFR1, PIF1, PIF3 and PIF4 were also detected. Without taking in account any possible differences in the amount of protein generated for bait and prey, the strongest interaction detected was PIF1 with PIF3, followed by PIF1 with PIF4. PIF1 and PIF4 interacted with similar strengths with HFR1. PIF3 and HFR1 had the weakest interaction but it is significantly different than the negative controls.

PIF1 and PIF3 interact with each other and bind to DNA as a heterodimer *in vitro*

To verify the interactions using an independent method, we selected PIF1 and PIF3 and cotranslated the two proteins using the TnT system as previously described (Toledo-Ortiz et al., 2003; Huq et al., 2002). GAD:PIF1 and GAD:PIF3 coimmunoprecipitated PIF1 and PIF3, respectively, and this coimmunoprecipitation was more efficient than the GAD control protein (Fig. 4.4 A). Moreover, GAD:PIF1 and GAD:PIF3 robustly coimmunoprecipitated PIF3 and PIF1, respectively. Taken together, these results confirm that the two proteins can form homodimers as well as heterodimers.

Since PIF1 and PIF3 can bind to DNA separately (Martinez-Garcia *et al*., 2000; Huq et al., 2004), we also investigated whether PIF1 and PIF3 can bind to the G-box DNA motif as a heterodimer. For this experiment, we used a truncated version of PIF3, which lacks the amino-terminal 308 amino acids but contains the bHLH domain, including the carboxy-terminal portion (ΔNPIF3) (Toledo-Ortiz *et al*., 2003), and GAD:PIF1, for better

separation of the heterodimer complex. The presence of a complex which migrates as an intermediate size band between the presumptive ΔNPIF3-ΔNPIF3 homodimer and GAD:PIF1-GAD:PIF1 homodimer complexes provides evidence that PIF1 and PIF3 can indeed form heterodimers that are capable of recognizing the G-box motif in a sequence-specific manner (Fig. 4.4 B). Thus, these two factors might control gene expression as both homo and heterodimers.

PIF1 and PIF3 function additively to repress photomorphogenesis under blue light

To understand the significance of the interactions among PIF family members and HFR1, *pif1pif3*, *pif1hfr1* and *pif3hfr1* double mutants were obtained. The *pif1pif3* double mutant presents the hypersensitive phenotype of *pif3* when grown in continuous red light (Figure 4.5 A). In the previous chapter it was discussed that the *pif1* mutant shows reduction of hypocotyl length and enhanced cotyledon opening under discontinuous blue light. These phenotypes were also present in the *pif3* mutant (Fig. 4.5 C-F). In addition, under continuous blue light *pif3* shows enhanced cotyledon opening (Figure 4.5 E) while in *pif1* it is not statistically different than the wild type. Figure 4.5 F shows that *pif3* mutant also presents more dramatic cotyledon opening than *pif1* in discontinuous blue light. The *pif1pif3* double mutant presented an enhanced reduction in hypocotyl length (Figure 4.5 D) and strikingly enhanced cotyledon opening phenotype in discontinuous blue light (Figure 4.5 F). The *pif1pif3* double mutant also presents these phenotypes in a reduced degree in continuous blue light. In both continuous and discontinuous light the greater difference between *pif1pif3* and *pif3* occurs at 2.1 μmoles/m^2s. These data suggest that PIF1 and PIF3 function additively to repress photomorphogenesis under blue light.

PIF1 and HFR1 function oppositely to regulate photomorphogenesis under all three light conditions applied diurnally

hfr1 seedlings show hyposensitive phenotypes in response to continuous far-red and blue light conditions (Fairchild et al., 2000; Duek and Fankhauser, 2003), while *pif1* displays hypersensitive phenotypes under discontinuous FR and blue light conditions (Oh et al., 2004; Castillon et al., 2009). In accordance with these data, *pif1hfr1* double mutant displayed phenotypes similar to *hfr1* single mutant under continuous far-red light (Figure 4.6 A) and blue light conditions (Figure 4.6 C). Moreover, *pif1hfr1* double mutant displayed intermediate phenotypes between *pif1* and *hfr1* single mutants under discontinuous far-red light conditions (Figure 4.6 B), presumably due to opposite phenotypes showed by these two mutants. Interestingly, *pif1hfr1* double mutant displayed phenotypes similar to *hfr1* single mutant under discontinuous blue light (Figure 4.6 F), suggesting that *hfr1* is epistatic to *pif1* under this condition.

Photoactivated phytochromes promote seed germination in response to red light, while seed germination is inhibited if a pulse of FR light is given to the seeds after imbibition. It has been shown that *pif1* mutants germinate in the darkness after a far-red light treatment due to the lack of repression of the gibberellin pathway (Oh et al., 2004, Oh et al., 2006, Oh et al., 2007). Figure 4.7 A shows that the *pif1hfr1* double mutants exhibit this same behavior as the *pif1* single mutant, suggesting that *pif1* is epistatic to *hfr1* in controlling seed germination in response to light.

The *hfr1* mutant also showed exaggerated shade avoidance responses when grown in a low R to FR light ratio (Sessa et al., 2005). *hfr1* mutant seedlings exhibited elongated inflorescence and petiole length and a small leaf area compared to wild type. *pif1* mutants did not exhibit these responses, but *pif1hfr1* double mutant demonstrated these responses with no significant difference to *hfr1* single mutant (Figure 4.7 B, C and D), suggesting that *hfr1* is epistatic to *pif1* in controlling these responses.

PIF3 and HFR1 function oppositely to regulate photomorphogenesis under continuous red, far-red and blue light conditions

In red continuous light *hfr1* mutant shows no significant phenotypic difference from wild type, and the *pif3hfr1* mutant thus takes the hypersensitive phenotype characteristic of *pif3* (Figure 4.8 A). In continuous far-red light conditions, *pif3* has no significant phenotype, and the *pif3hfr1* mutant thus takes the hyposensitive phenotype characteristic of *hfr1* under these conditions (Figure 4.8 B).

Because *pif3* single mutant showed hypersensitive phenotype in response to both continuous and discontinuous blue light and *hfr1* single mutant displayed hyposensitive phenotypes under these conditions, we analyzed the behavior of the *pif3hfr1* double mutant (Figures 4.8 C-F). Results show that the cotyledon angle phenotypes of the *pif3hfr1* double mutant are largely similar to the *pif3* single mutant, suggesting *pif3* is epistatic to *hfr1* under these conditions. *pif3* also showed slightly shorter hypocotyl length compared to wild type under both continuous and discontinuous blue light conditions. However, *pif3hfr1* displayed almost wild type phenotype under these conditions, presumably due to the opposite behavior of *pif3* and *hfr1* single mutants under these conditions.

DISCUSSION

In other systems, bHLH transcription factors function as homodimers and heterodimers (Littlewood and Evans, 1998). In this study, we provide biochemical, genetic and photobiological evidence that plant bHLH proteins also function as homodimers and heterodimers in controlling specific biological pathways in Arabidopsis. To identify bHLH interaction partners, a yeast two-hybrid screening strategy was employed. Full-length PIF1 possesses strong transcriptional activation activity in yeast. Deletion analyses demonstrated that the first 150 amino acids of PIF1 contain the

transcriptional activation activity of PIF1. These data are consistent with the previous report that this region of PIF1 is also necessary and sufficient for transcriptional activation activity *in planta* (Shen et al., 2008). Several clones were identified using the longest fragment (C328) that lacked the transcriptional activation activity. The bHLH factors identified from this screen include PIF3, PIF4, HFR1 and PIF1 itself, suggesting that PIF1 also homodimerizes. These bHLH proteins have been previously implicated in the phytochrome-mediated light signaling pathways. These data suggest that PIF1 can heterodimerize with these bHLH proteins and that these interactions are functionally relevant in controlling photomorphogenesis.

A quantitative yeast two-hybrid interaction assays revealed that all the possible heterodimer combinations among PIF1, PIF3, PIF4 and HFR1 are formed. Bait constructs with full-length PIF1, PIF3 and PIF4 showed transcriptional activation capacity in the absence of a prey. Full-length HFR1 fused to GAL4 binding domain, did not activate transcription by itself in a yeast two-hybrid experiment, indicting that HFR1 being an atypical bHLH protein might lack the capacity to activate transcription by itself or through homodimer formation. This is an interesting finding since it opens the possibility that when HFR1 heterodimerizes with PIFs it might hinder the PIFs' transcriptional activation capacity. This suggestion is supported by the fact that in the quantitative interaction assays among PIFs and HFR1, the interactions among PIF1-HFR1, PIF3-HFR1 and PIF4-HFR1 show weaker transcriptional activation capacity than PIF1-PIF3 and PIF1-PIF4. A caveat to this assumption is that the relative strengths of the interactions could be affected by the differential expression levels of the various constructs involved.

Previously, we have shown that among the PIF family members, only PIF1 functions negatively to regulate blue light signaling in Arabidopsis (Castillon et al., 2009). This study shows that *pif3* seedlings also exhibit hypersensitive phenotypes including shorter

hypocotyls, larger cotyledon area and enhanced cotyledon opening when grown under continuous and discontinuous blue light conditions. The open cotyledon phenotype under discontinuous blue light is especially strong at the low fluences of blue light (0.05 and 0.21 μmoles/m^2s) (Figure 4.5 E and F). Because PIF3 protein level cycles under light/dark cycle conditions (Monte et al., 2004), it is possible that the enhancement of this phenotype in discontinuous blue light in comparison to continuous blue light is due to the effect of the accumulation of PIF3 in the periods of darkness. In wild type seedlings PIF3 accumulates in the darkness repressing the photomorphogenic responses, and causing closed and smaller cotyledons as well as longer hypocotyls. *pif3* mutants lacking this repression would appear with more open and expanded cotyledons and shorter hypocotyls. These data suggest that other PIF family members might also function under blue light conditions.

PIFs function negatively and HFR1 function positively in regulating light signaling pathways (Castillon et al., 2007; Duek and Frankhauser, 2003). To investigate the functional significance of hetreodimerization among the bHLH proteins involved in the light signaling pathways, double mutant combinations were created. Phenotypic characterization revealed that PIF1-PIF3 heterodimer functions additively, while PIFs and HFR1 function to antagonize each other in regulating photomorphogenesis. For example, *pif1pif3* double mutant showed additive phenotypes in promoting photomorphogenesis under blue light conditions (Figures 4.5 D and F). Figure 4.5 F shows *pif1pif3* double mutant present enhancement of cotyledon expansion especially at low intensity of blue light (2.1 mmoles/m^2s). The *pif1hfr1* and *pif3hfr1* mutants showed the characteristic phenotypes of *hfr1* under conditions where PIFs have reduced functions. HFR1 functions as a positive regulator of photomorphogenesis, so the *hfr1* mutant exhibits hyposensitivity under far-red and blue light conditions. In the case of the double mutants the lack of the positive regulator is epistatic over the absence of the negative regulator, since the double mutants show *hfr1* phenotype.

Taken together these results suggest that a network of interactions between PIF1, PIF3, PIF4 and HFR1 occurs in which all combinations are possible. PIFs are more abundant in the dark-grown seedlings and are degraded in response to light signals (Castillon et al., 2007). By contrast, HFR1 is degraded in the dark and is stabilized by light signals. Both PIFs and HFR1 are constitutively nuclear localized factors. Therefore, there appears to be a gradient of these factors present in the same subcellular compartment in seedlings during transition from dark to light. Although light induces degradation of PIFs to remove their negative regulatory roles during photomorphogenesis, most PIFs are present at a small level under prolonged light conditions, suggesting that the light-induced degradation may not be enough to remove their function completely. Because the positively acting bHLH protein, HFR1, can heterodimerize with all the negatively acting PIFs, it is possible that the function of this heterodimerization among PIFs and HFR1 is to remove the residual PIF functions to promote photomorphogenesis. Further biochemical experiments are necessary to understand the mechanisms by which PIFs and HFR1 function antagonistically.

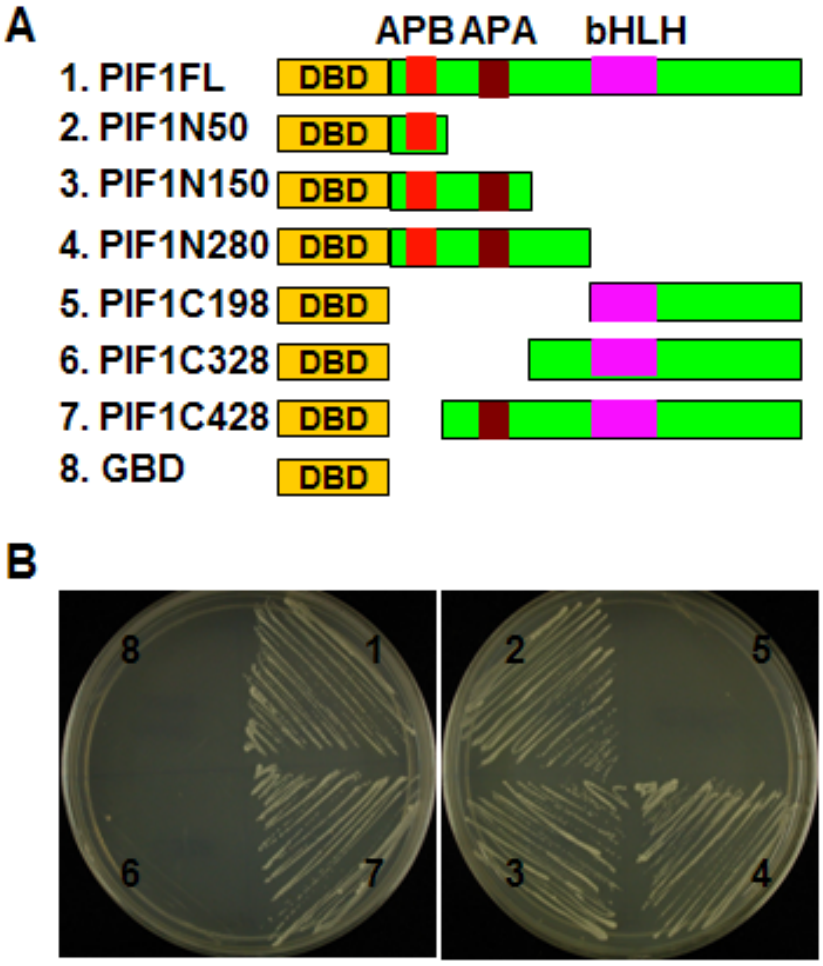
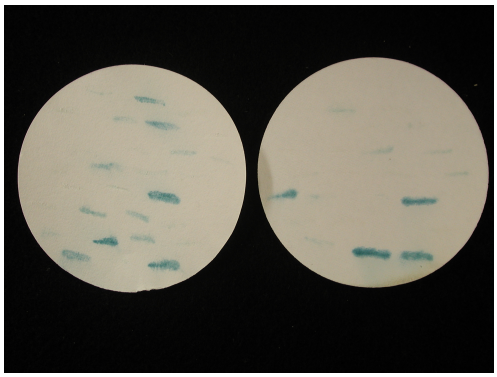


Figure 4.1: PIF1s transcriptional activation domain is located in the N-terminal 150 amino acids. A) Diagram of the six deletion constructs. B) Deletion constructs C328 and C198, lacking 150 amino acids from the N-terminus, lack transcriptional activation activity. Yeast growth in media without histidine show the activation of the *HIS3* reporter gene in all constructs except C328 and C198. Empty GBT9 was used as negative control.

A

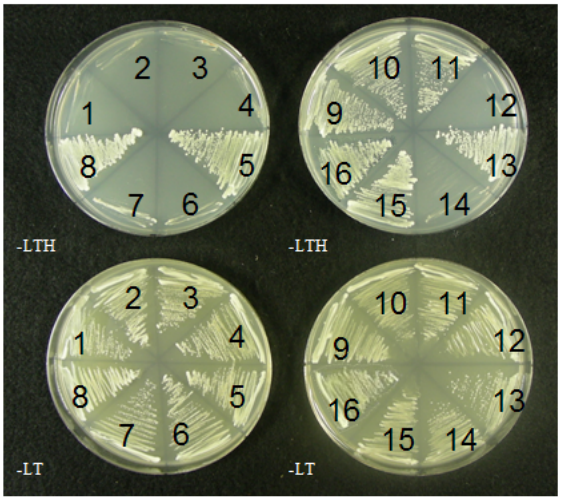


B

Identification number	Protein	Number of times picked up in screen
At1g09530	PIF3	2
At1g02340	HFR1	7
At2g20180	PIF1	1
At5g67320	WD-40 repeat protein	1
At4g34110	Poly-A binding protein (PABA2)	8
At1g01090	E1 subunit of pyruvate dehydrogenase	2
At2g27020	Proteasome alpha subunit G	3
At3g25840	Chloroplast kinase	1
At1g78260	RNA recognition motif protein	2
At3g46780	Unknown function	1
At4g33740	Unknown function	1

Figure 4.2: Results for the yeast two-hybrid screen, using PIF1 deletion construct C328 as a bait. A) LacZ assays showing β-galactosidase activity. B) Table of interactors picked up in screen.

A



Bait construct: Prey construct

1 GBT9 : GAD424
2 PIF1-GBT : GAD424
3 GBT9 : HFR1-GAD
4 HFR1-GBT : GAD424
5 PIF1-GBT : HFR1-GAD
6 PIF3-GBT : GAD424
7 GBT9 : PIF3-GAD
8 HFR1-GBT : PIF1-GAD
9 PIF1-GBT : PIF3-GAD
10 PIF3-GBT : PIF1-GAD
11 HFR1-GBT : PIF3-GAD
12 GBT9 : PIF3-GAD
13 PIF3-GBT : HFR1-GAD
14 GBT9 : PIF4-GAD
15 HFR1-GBT : PIF4-GAD
16 PIF1-GBT : PIF4-GAD

B

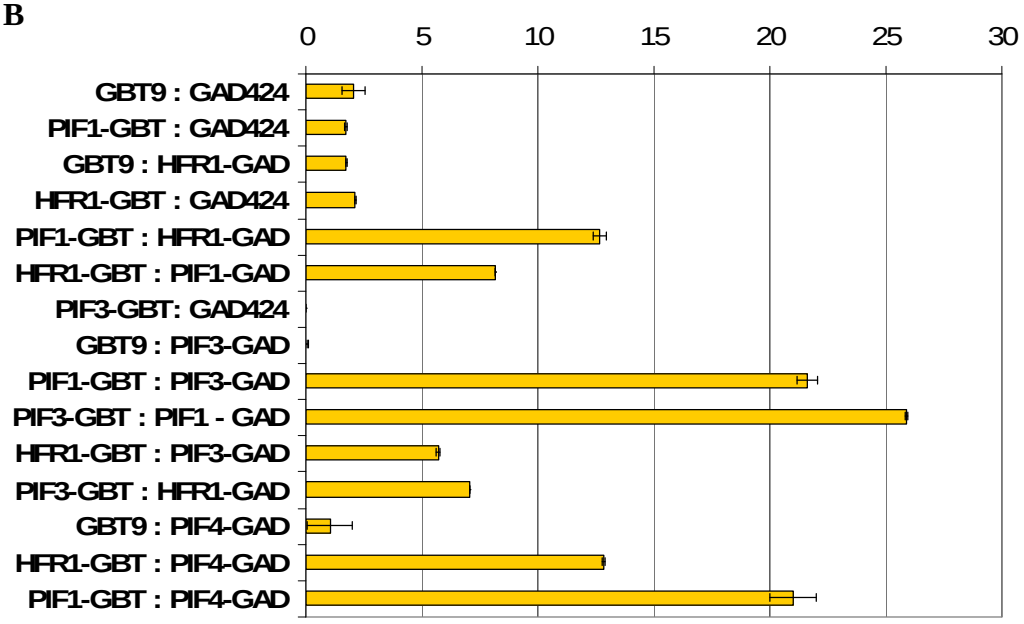


Figure 4.3: PIF1 interacts with PIF3 and PIF4, and HFR1 interacts with PIF1, PIF3 and PIF4. A) Yeast growth in media lacking leucine, tryptophan and histidine shows the interactions among PIFs and HFR1. B) Quantification of the interactions between PIF1, PIF3, PIF4 and HFR1. LacZ assays were performed in triplicate and the data represent mean $\pm$ SE. β-Galactosidase units are M.U., Miller units.

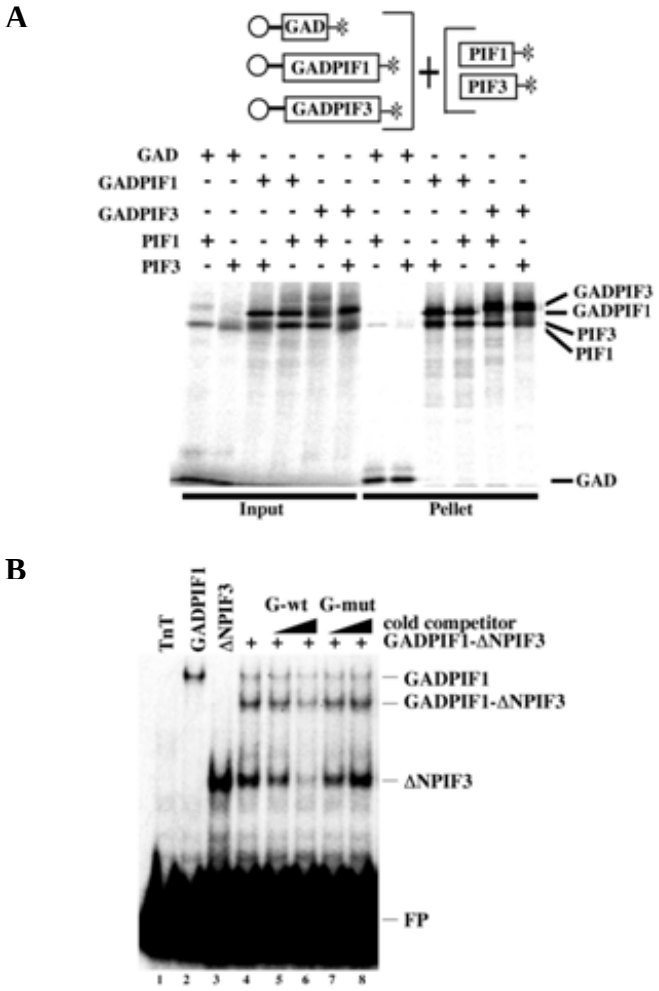


Figure 4.4: PIF1 heterodimerizes with PIF3. (A) PIF1 interacts with PIF3 *in vitro*. (Top) Schematic representation of the baits (left) and the preys (right) used for this experiment. (Bottom) The gel photograph shows the input and the pellet fraction. Full-length PIF1 cDNA either alone or fused to GAD was used for this coimmunoprecipitation assay, according to Toledo-Ortiz *et al*., 2003. All proteins were synthesized as ^{35}S-methionine labeled products in TnT reaction. GBD and GAD are GAL4 DNA binding and activation domains, respectively. (B) PIF1 binds to the G-box as a heterodimer with PIF3. GAD:PIF1 and a truncated DNPIF3 clone were coexpressed in TnT, and 1 ml of this TnT mix was used for DNA binding. GAD:PIF1 and ΔNPIF3 were also expressed in TnT separately and used to bind to the G-box DNA as homodimers. Binding conditions are described in Toledo-Ortiz *et al*., 2003. Data obtained by Enamul Huq.

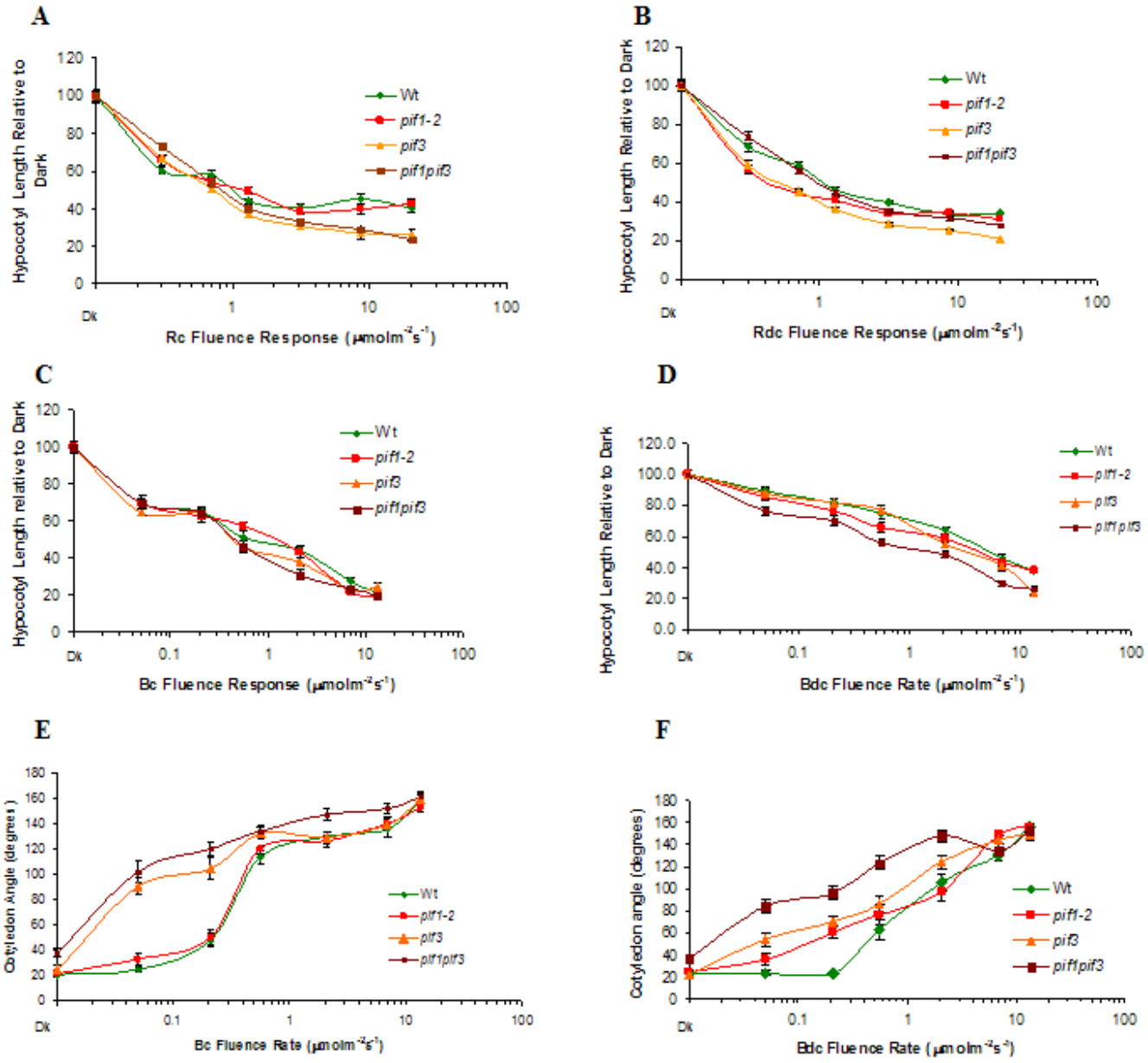


Figure 4.5: Phenotypical characterization of *pif1pif3* double mutant. Fluence-rate response curves of mean hypocotyl lengths (A-D) and cotyledon angles (E and F) and of wt (Col-O), *pif1-2, pif3* and *pif1pif3* grown for four days under either continuous red light (A), diurnal red light (B), continuous blue light (C and E) or diurnal blue light conditions (D and F). Hypocotyl lengths were normalized by setting the dark values to 100. Data are presented as mean $\pm$ SEM ($n \geq 30$, three replicates).

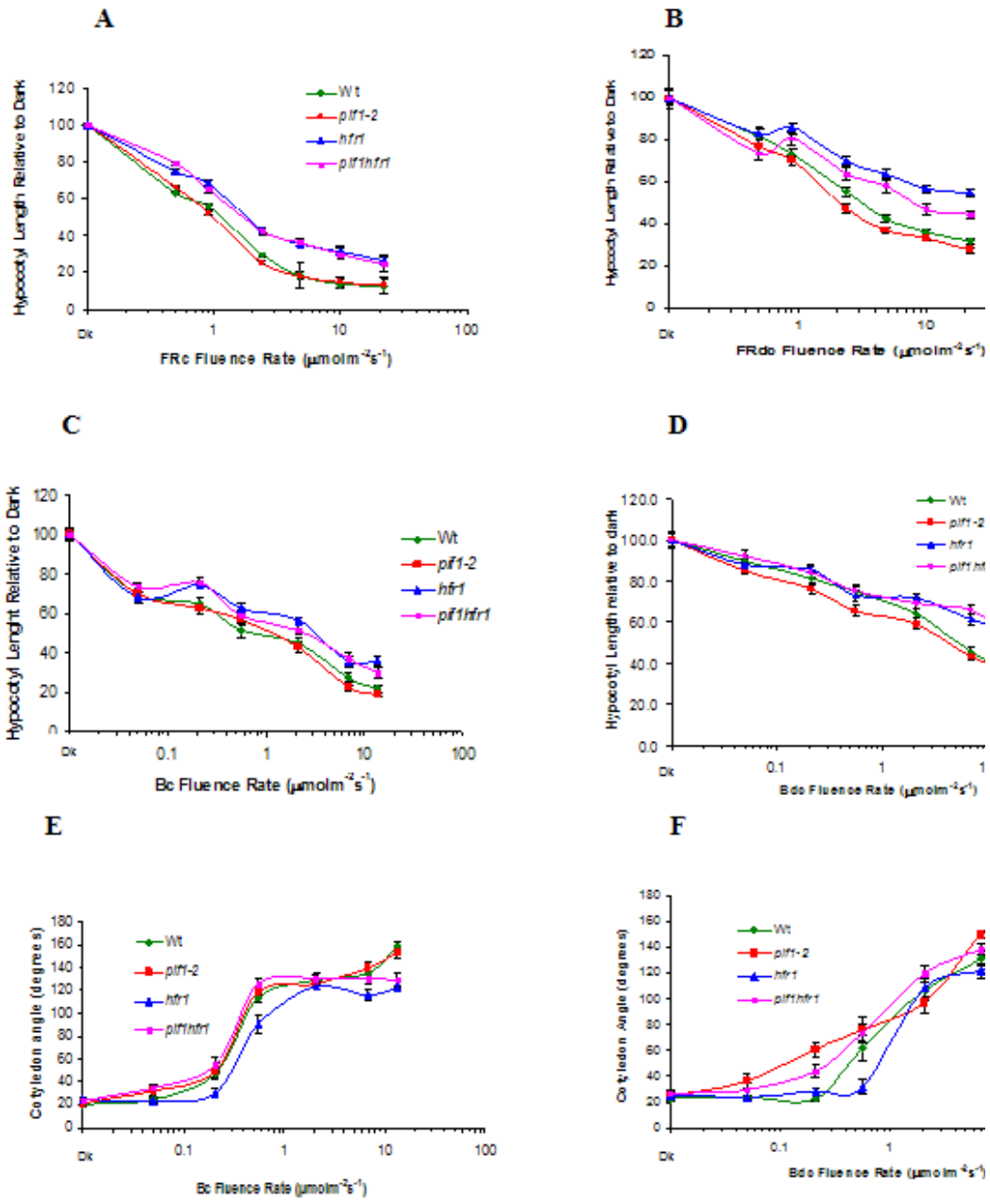


Figure 4.6: Phenotypical characterization of *pif1hfr1* double mutant. Fluence-rate response curves of mean hypocotyl lengths (A-D) and cotyledon angles (E and F) and of wt (Col-O), *pif1-2*, *hfr1* and *pif1hfr1* grown for four days under either continuous far-red light (A), diurnal far-red light (B), continuous blue light (C and E) or diurnal (12h light/12h dark) blue light conditions (D and F). Hypocotyl lengths were normalized by setting the dark values to 100. Data are presented as mean $\pm$ SEM ($n \geq 30$, three replicates).

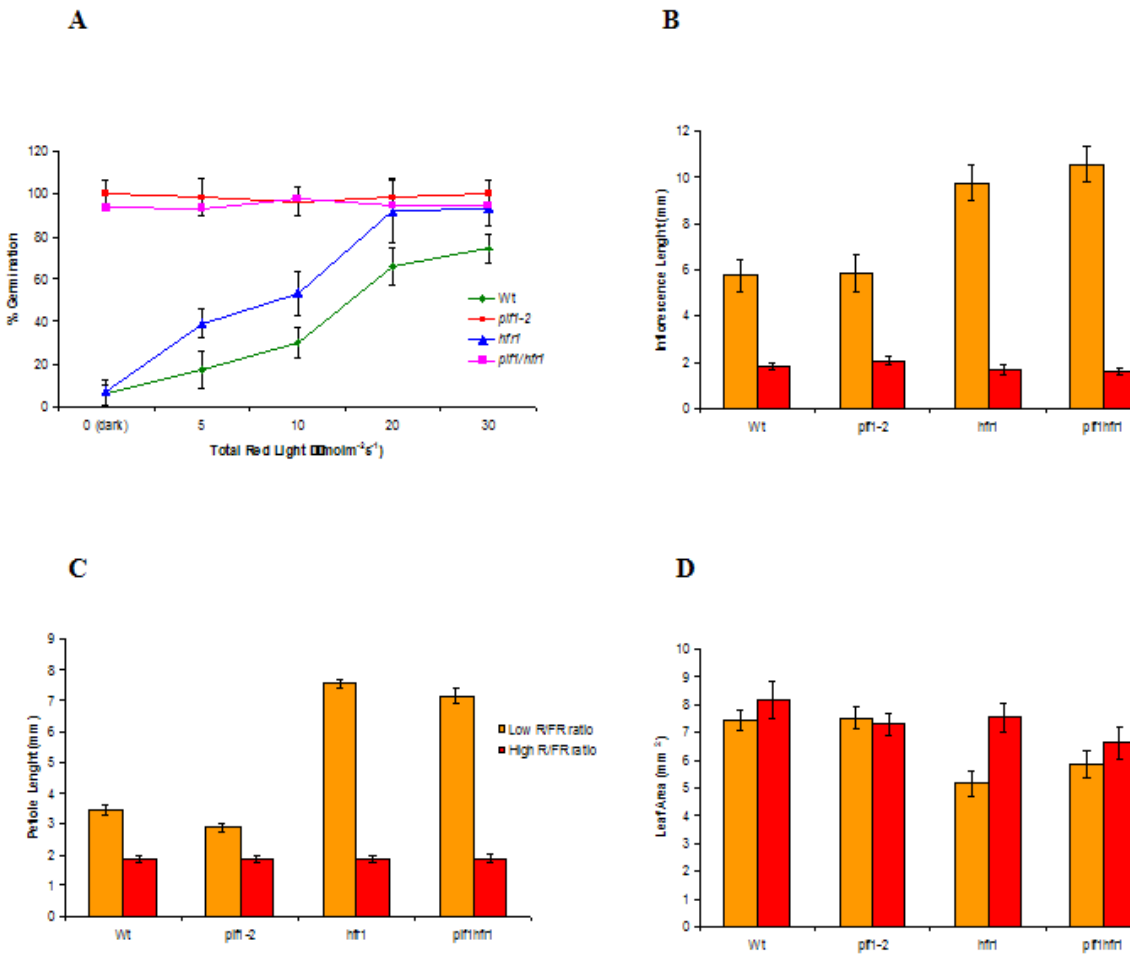


Figure 4.7: A) *pif1hfr1* double mutants germinate in the darkness after exposure to far-red light in the phyB mediated germination assay. *pif1hfr1* double mutants show shade avoidance responses similar to *hfr1*. *pif1hfr1* shows elongated inflorescence length (B) and petiole length (C), as well as reduced leaf area (D) under low red to far-red ratios. Data are presented as mean $\pm$ SEM ($n \geq 30$, three replicates).

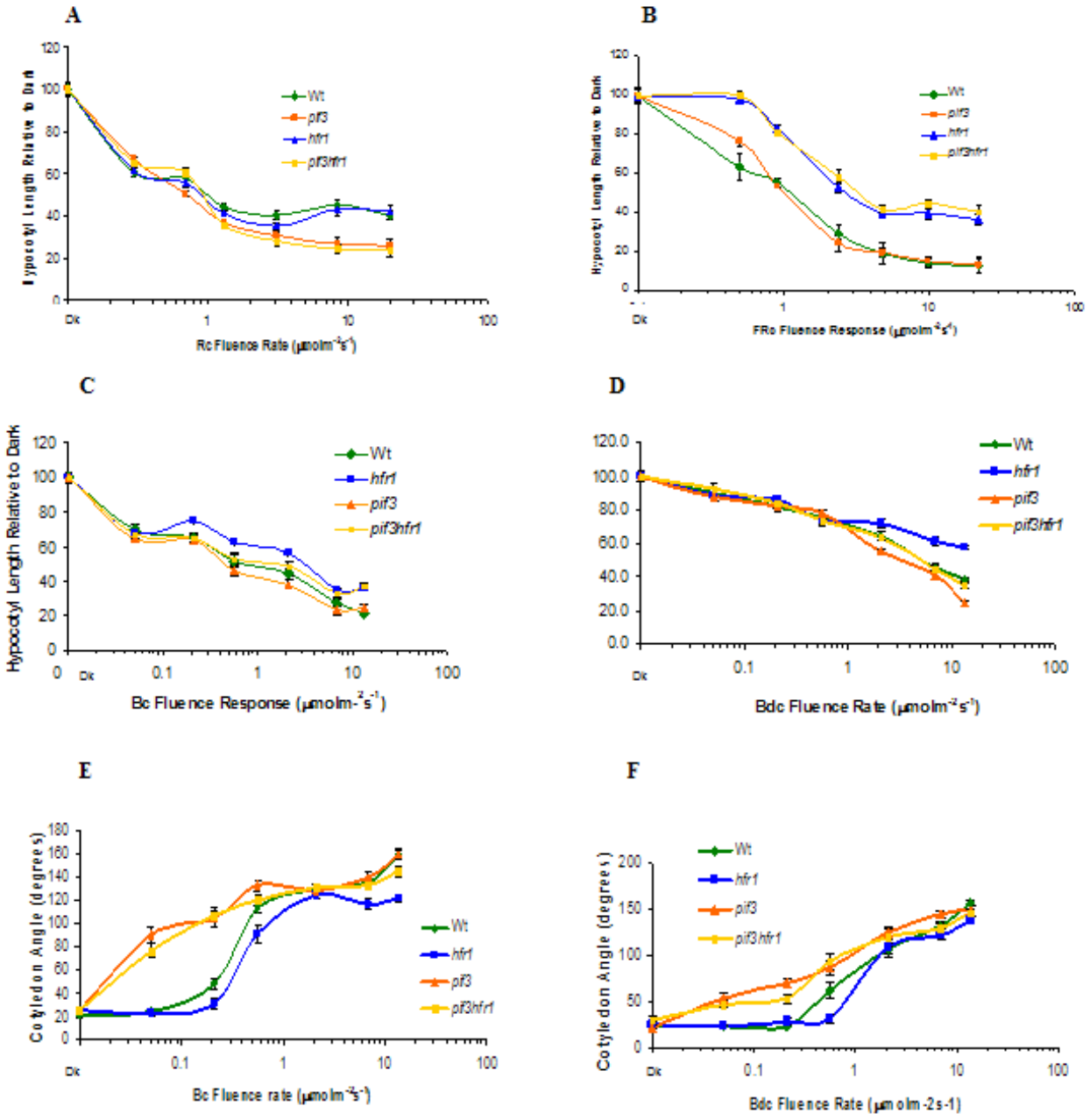
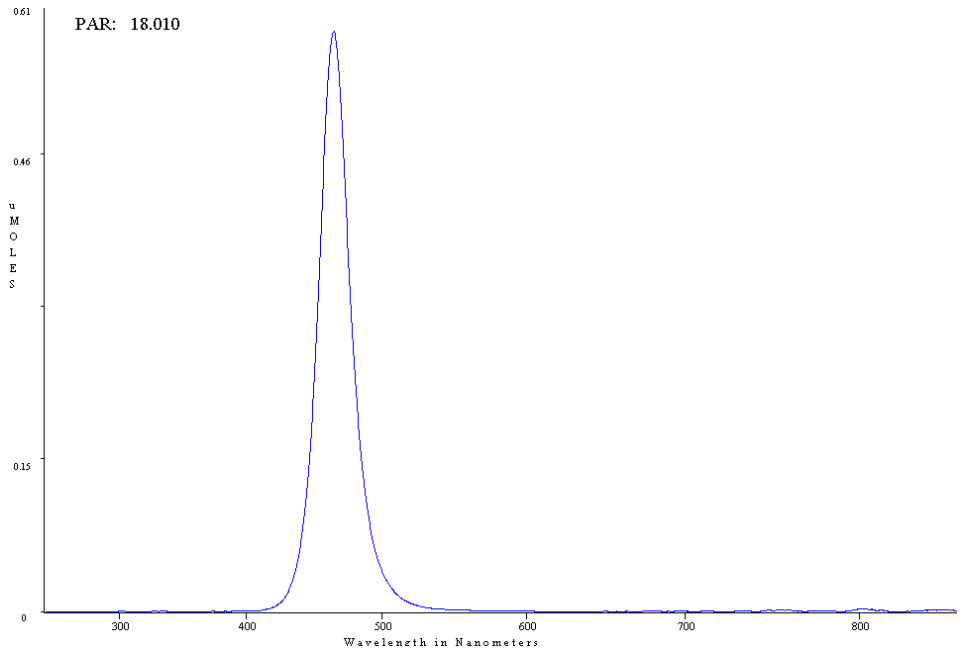


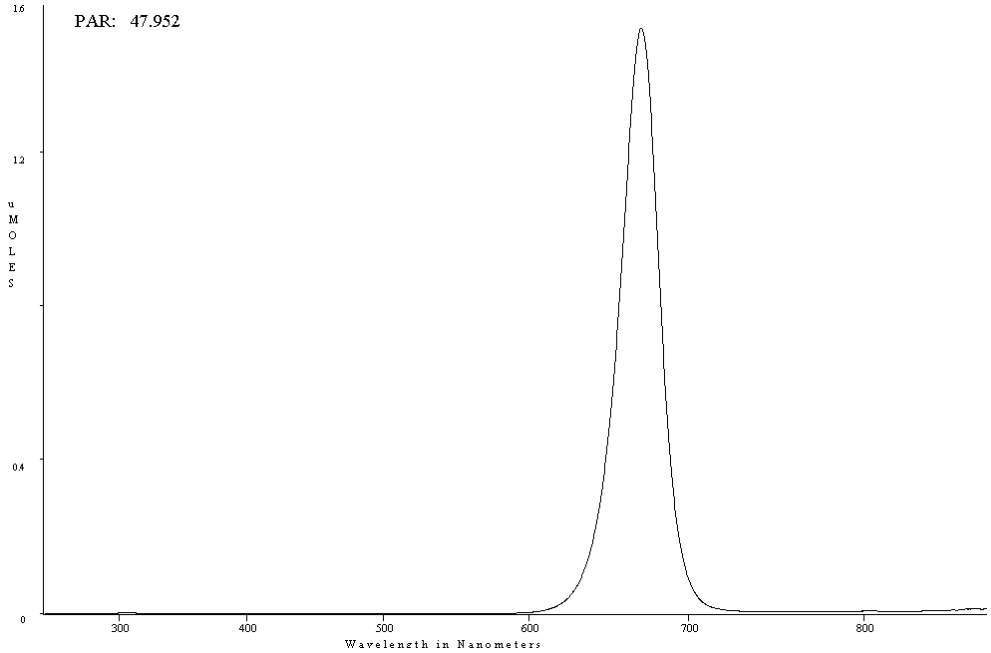
Figure 4.8: Phenotypical characterization of *pif3hfr1* double mutant. Fluence-rate response curves of mean hypocotyl lengths (A-D) and cotyledon angles (E and F) and of wt (Col-O), *pif3*, *hfr1* and *pif3hfr1* grown for four days under either continuous red light (A), continuous far-red light (B), continuous blue light (C and E) or diurnal (12h light/12h dark) blue light conditions (D and F). Hypocotyl lengths were normalized by setting the dark values to 100. Data are presented as mean $\pm$ SEM ($n \geq 30$, three replicates)

APPENDIX 1

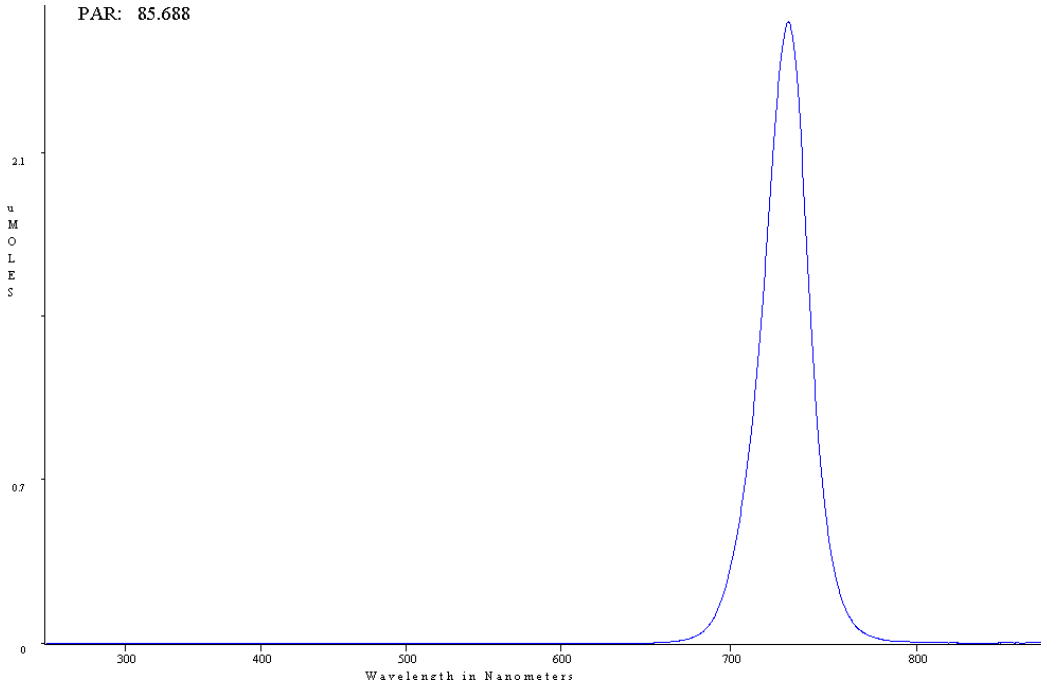
Wavelength specificity of the LED light sources used for experiments in this study.



A) Blue Light, peak at approximately 460 nm.



B) Red light, peak at approximately 660 nm.



C) Far-red light, peak at approximately 720 nm.

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